Dr. Lowell P. Bush

Philip Morris Professor

PHILIP MORRIS
INCORPORATED

1992-1993

College of Agriculture
University of Kentucky
Lexington, Kentucky

1992-1993 PHILIP MORRIS PROFESSORSHIP REPORT

Lowell P. Bush

University of Kentucky

EXECUTIVE SUMMARY

During 1992 and 1993 the research of Dr. Lowell Bush - Philip Morris Professor of Agriculture, Department of Agronomy, University of Kentucky - emphasized the demethylation of nicotine and the accumulation of tobacco-specific nitrosamines (TSNA) and TSNA precursors in leaf tissue. In addition research on the symbiotic mutalism between host and fungal endophyte for saturated aminopyrrolizidine alkaloid metabolism was conducted. *N. glauca*, an anabasine accumulator, and *N. africana*, a nicotine/nornicotine accumulator, rapidly demethylated nicotine in vivo when nicotine was fed to leaves. Demethylation in lamina was much greater than in midribs. Tetcyclasis inhibited nicotine demethylation suggesting P-450 involvement in demethylation. In vitro demethylation was most associated with microsomes and required NADPH and oxygen. Enzyme activity was maximal at pH 7.0-7.5 and 30°C. Typical rates of nornicotine formation was 10 to 50 pmol min^{-1} (mg of protein)$^{-1}$, while Vmax and the apparent K_m(nicotine) were 105 pmol min^{-1} and 51 μm, respectively. In vitro nicotine demethylation activity was inhibited by antichrome P-450 reductase but not by CO.

Nitrate accumulation was greatest in the midrib and decreased toward the margins of air cured leaves. Nitrite accumulation was greatest in the basal portion of the midrib, but uniformly high in most other parts of the leaf. Accumulation of nitrite did not parallel concentration of nitrate. The basal portion of the midrib contained greatest accumulation of TSNA but the apical portion of the midrib had the lowest concentration of any leaf tissue. In these studies there was a better relationship between nitrite nitrogen and TSNA than between alkaloids and TSNA. In genotypes for different alkaloid accumulation alkaloid content had been the better predictor of TSNA accumulation.

Based on these results investigations were initiated to determine the significance of leaf microflora on nitrite and TSNA during of air curing. These investigations are on-going and details will be provided as they are completed.

Much collaboration with other faculty, postdoctoral fellows, graduate students, technical support personnel and visiting scientists was essential to the research efforts. Two long term visiting scientists were (1) Cui Mingwu, a CORESTA study recipient who has become a graduate student working on MH residues and float transplant production and (2) Wei Xiaochen, also a CORESTA study grant recipient, working on the mechanism of nicotine demethylation and nitrosonornicotine formation.

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Noteworthy Professional Recognition and Activities

- Will complete a 5 year elected term to the Policy Committee of the Tobacco Chemists' Research Conference as Chairman, 1993-94.
- Invited and presented three seminars on tobacco science at Henan Agricultural University, Zhengzhou, PCR
- Invited to present and write a chapter on Saturated Pyrrolizidine Alkaloids to the 1st Intl. Symp. on Acremonium/Grass Interactions, published 1993.
- Invited to present a paper on Nicotine Metabolism to IUTOX meeting in Salsomaggiore Terme, Italy, published 1993.
- Invited to moderate the Chemistry session at the 2nd Intl. Symp. on Acremonium/Grass Interaction, Palmerston North, NZ. 1993.
- Invited with H.R. Burton to present a paper on Accumulation of tobacco-specific nitrosamines during curing and aging of tobacco at the symposium on Nitrosamines and N-Nitroso Compounds: Chemistry and Biochemistry. presentation 1993.
- Invited to present and write a chapter on Intrinsic Chemical Factors in Forage Quality, presentation 1994.
- Invited with M.R. Siegel to write a chapter on Importance of Endophytes in Forage Grasses for book titled Biotechnology of endophytic fungi of grasses.
- Invited with M.R. Siegel to write a chapter on Altered Host Metabolism in Grass/Endophyte Associations: Endophyte Toxin Production for The Mycota.
- Graduate students, post-doctoral fellows or Professor Bush made presentations at Tobacco Chemists' Research Conference 1992 and 1993, Tobacco Workers Conference 1993, CORESTA Symposium 1992, American Society of Agronomy 1992 and 1993.

TEACHING activities have included:

- An undergraduate course each year on Environment, Food Production and Society in the US
- A portion of Plant Biology, an undergraduate course with ~200 students per section.
- A portion of a graduate course on Tobacco
- Advising Mr. Cui Mingwu of China National Tobacco Company, a former CORESTA study grant recipient in the laboratory, presently a MS student working on tobacco float transplant production
- Mr. Wei Xiacohen is a visiting scientist in the laboratory from the Zhengzhou Tobacco Research Institute, PRC and a CORESTA study grant recipient.

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Research Support

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Distribution of Tobacco Constituents in Tobacco Leaf Tissue: Tobacco-Specific Nitrosamines, Nitrate, Nitrite, and Alkaloids

The accumulation of tobacco-specific nitrosamines (TSNAs) during air-curing has been of considerable interest for the past several years (Burton et al., 1989a,b; Peng, 1990; Djordjevic et al., 1989). This has been a result of the report that several TSNAs induce malignant tumors in mice, rats, and hamsters (Hoffman and Hecht, 1985). The TSNAs are derived via nitrosation of secondary and tertiary alkaloid amines which are present in tobacco. Even though there have been numerous reported studies concerned with the accumulation of nitrosamines in tobacco, there sometimes have been conflicting conclusions. Brunnemann et al. (1983) reported there was a direct relationship between nitrate concentrations and TSNAs in tobacco. Djordjevic et al. (1989) reported there was a significant, positive correlation between alkaloid content in tobacco and TSNA accumulation; however, they found no relationship between nitrate and TSNA content. Recent studies by Burton et al. (1989 a,b, 1990) have shown there was a direct relationship between TSNA levels and nitrite concentration in tobacco. They also reported there was no relationship between alkaloid or nitrate content and nitrosamine concentration. Analyses of lamina and midrib tissue showed that midrib contained higher TSNA levels than lamina. Also, the midrib contained significantly higher concentrations of nitrite nitrogen. Djordjevic (1989) reported midribs contained lower TSNA concentration in comparison to lamina.

Because of the confusion generated by published data, it was decided to investigate nitrosamine accumulation in greater detail. The previous studies involved the analysis of lamina and veinal tissue of the whole tobacco leaf. It was proposed that TSNAs may accumulate in higher or lower concentration in certain locations of the leaf on the basis of the reports that nicotine has been shown to accumulate in higher concentrations on the periphery of the lamina tissue (Andreadis et al., 1939; Gay et al., 1984). Neurath and Emke (1964) reported nitrate accumulation was highest in the midvein and lowest at the tip of green tobacco leaf. Also, Jenkins (1986) reported that ions, e.g., Ca^{2+}, Mg^{2+}, Na^+, K^+, Cl^-, and Br^-, were nonuniformly distributed throughout

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the laminar tissue. The purpose of this study was to segment lamina and midvein tissue and to determine if there was nonuniform distribution of TSNAs, nitrate, and nitrite, and alkaloids in these tissues and to determine if there were significant correlations between levels of these constituents. These data would aid in determining which constituents have greatest influence on the accumulation of TSNAs.

Experimental Procedures

A dark tobacco type (Ky 171), which is used primarily for smokeless tobacco, was grown at the University of Kentucky Agricultural Experiment Station Farm in 1990 using standard cultural practices for the production of dark tobacco. The tobacco was stalk-cut and air-cured in a conventional curing barn. After curing, four leaves were removed from the top third of five plants. The leaves were then placed on a grid and sectioned into 7 cm long x 4 cm wide segments along the length of the leaf (Figure 1). There were 10 segments along the length of the leaf, and there was a maximum of 8 segments across the width of the leaf at its widest point. The midrib was segmented in 7-cm lengths. Lateral veins were separated from the lamina segments and identified by the corresponding lamina segment number. To reduce the number of samples and have sufficient quantities of tobacco for analyses, the lamina segments were combined with their mirror image segments (Figure 1). The lateral veins were combined in the same manner. Weights for all leaf segments were determined after drying. After the samples were ground in a small laboratory mill, the samples were stored at -20°C until analyses.

Nitrate was analyzed using a Technicon autoanalyzer and Technicon procedure (Technicon Industrial Method 100-70W/B). Nitrite concentrations were determined using a procedure described by Crutchfield and Burton (1989). Extraction of tobacco for analyses of TSNAs employed a procedure reported by Burton et al. (1988) for the analyses of acylated nornicotine. Because of the potential carcinogenis of the TSNAs, all extractions and concentrations of the extracts were carried out in laboratory hoods. Individual TSNAs from the extract were quantified on a Thermedics Inc. TEA Model 543 analyzer coupled to a Hewlett-Packard Model 5890 gas chromatograph equipped

with a 50 M x 0.25 mm capillary column coated with a 0.25-μm DB-5 liquid phase. The chromatographic conditions and calculation of response factors have previously been reported (Peng, 1990). Three-dimensional graphical presentation of data was obtained using the Surfer program purchased from Golden Software Inc. All graphical data are presented on a 65 x 73 grid.

Results and Discussion

Data from leaf segments for the weight, nitrate and nitrite, and individual alkaloids are presented in Table I. The leaf segments correspond to those shown in Figure 1. The lowest number was from the tip of the leaf. Increasing letters A-D were from the midvein to the outer edge of the leaf. Because of the insufficient weight of the lateral vein, leaf segments were combined within Arabic numeral. For example, lateral veins from leaf segments 1A, 1B, 2A, 2B, and 2C were combined to obtain sufficient quantities of material for subsequent analysis of individual alkaloids, nitrate N, nitrite N, and individual TSNAs. Even then there was sufficient sample in several combined segments for only analyses of TSNAs. In general, the weights of the lamina segments increase from the base of the leaf to the tip of the leaf and from the midrib toward the outer edge of the leaf. The outer edge of the leaf segment contains lower net weight, but this was because a full 28-cm² section was not obtained due to the geometry of the leaf. The weight of the midvein segments decreased from the base to the tip of the leaf. Approximately 50% of midrib was contained in segments 8-10, which is only 30% of the total length of the midvein.

Nitrate N and Nitrite N Concentrations in Leaf. In lamina (Table I), nitrate nitrogen is highest in the middle (segment 6) of the leaf, whereas, it is lower at the tip and base of the leaf. The tip of the leaf contains the lowest concentration of nitrate N. Nitrate levels are highest next to the midvein and lowest at the outer edges of the lamina. The highest concentration of NO_3^- N is in the sixth segment of the leaf. This was not predicted, since it was expected to decrease from the base to the tip of the leaf. This was based on the premise that since nitrate concentration is always highest in the midvein of the leaf (Burton et al., 1983, 1989b) and that through movement of

nitrate from the root to the lamina, a differential concentration of nitrate along the length of the leaf would be expected. Data for midvein also show that nitrate concentration is highest in the middle (Table I) and not at the base of the midvein. It is interesting to note nitrate is highest in the sixth segment and is also highest in the sixth of the lateral veinal tissue, indicating the highest concentration of nitrate in the midsection of the total leaf. To obtain the best overview of differences in the levels of nitrate in the leaf, data were graphically presented as a surface map. The data for nitrate using a surface map are shown in Figure 2. The X,Y dimensions are the length and width of the leaf, respectively, and the Z dimension is the concentration for nitrate (micrograms per gram) generated from the data for the leaf segment values including both lamina and midvein. Fitting these data points gave the approximation of the concentration for nitrate in the lamina. Because grid plots used a rectangular grid and the leaf geometry was oval, we forced the values outside the leaf boundary to 0. This plot clearly showed the tip of the leaf (segment 1) and the periphery contained the lowest level of nitrate, whereas the midrib (0) contained the highest concentration of nitrate N.

The concentrations of nitrite N did not parallel the concentration of nitrate in the cured lamina (Table I and Figure 3). Nitrite N ranged from 1 to 4 $\mu g\ g^{-1}$, which was as much as 2000 times lower than nitrate N. It was apparent only a small amount of nitrate was converted to nitrite under normal air-curing conditions. Concentration of nitrite was highest at the base of the leaf and lowest at the tip of the leaf (Figure 3). The concentration of nitrite in the midrib (segments 1-6) was lower than in the surrounding lamina segments, even though the midrib contained the highest levels of nitrate. This indicates that factors other than nitrate concentration influenced nitrite accumulation. The greater accumulation of nitrite at the base of the leaf can be explained by the observation that dry-down of lamina is slower at the base of the leaf. Maintenance of higher moisture content during death of the cells should support greater leaf and microbial metabolism, which can result in enhanced reduction of nitrate to nitrite (Burton et al., 1988, 1989b). At present, it would seem that nitrate reductase is coming from microbial metabolism. However, this cannot be shown by the

data from this study. From Figure 3 it is apparent that the midrib from the base of the leaf contains the highest concentration of nitrite.

Distribution of Alkaloids in Leaf. Data of the individual alkaloids in general show lamina contains the highest concentration of individual alkaloids, followed by the lateral veins and then midvein (Table I). It has been well documented that midvein tissue contains the lowest levels of alkaloid (Burton et al., 1983, 1988; Djordjevic et al., 1989; Gay et al., 1984; Andreadis et al., 1939). The accumulation of alkaloids increases from the center of the leaf to the outer edges. This is in agreement with data reported by Gay et al. (1984) using a single leaf of a greenhouse-grown burley tobacco variety. The data can be best visualized by using a surface plot of the analytical data. The nicotine values in Figure 4 are weighted averages of lamina plus lateral veinal tissue. They were calculated by using weighted averages of the lamina and midvein values obtained from the gas chromatographic analyses. They were included in the figure since leaf is generally separated into only lamina and midvein for most analyses. The data from this surface plot clearly show that nicotine concentration is greatest on the periphery of the leaf. The concentration of nicotine does decrease from a maximum in segment 7 to a minimum in segment 1 of the lamina. Analyses of the lateral vein for nicotine show that this alkaloid increased toward the outer edges of the leaf (Table I). Along the midrib the concentration of nicotine increased from the base (segment 10) to the tip of the leaf (segment 1). Nornicotine distribution in the leaf is similar to that of nicotine (Figure 5) since the midvein contains the lowest concentration of this alkaloid and nornicotine is highest at the outer edge of the lamina. However, the difference in nornicotine concentration between segment 1 and segment 4 (Figure 5) is not as great as that for nicotine (Figure 4). The other minor alkaloids (myosmine, anabasine, and anatabine) have the same distribution pattern as nicotine (figures not presented). This would be predicted since one would expect their physical and chemical characteristics to be similar to those of nicotine and, therefore, their distribution within the leaf to be the same as that of nicotine. It should be noted that the concentration for the individual alkaloids is greatest in segments 7 and 8. This

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is the section where the leaf has its greatest width. The reasons for the higher accumulation within these segments are not known.

Since the distributions of individual alkaloids, nitrate, and nitrite, within the lamina were quite different, it should be possible to discern if the concentrations of these constituents within the leaf are correlated with the concentration of the TSNAs in tobacco. One would predict that alkaloids and nitrite would influence nitrosamine accumulation since they are both necessary intermediates for nitrosamine formation.

Distribution of Tobacco-Specific Nitrosamines. Data for distribution of nitrosamines in leaf are presented in Table II. In lamina the concentration of nitrosamines was lowest at the tip of the leaf and then increased to maximum levels at the base of the leaf. Also, nitrosamine concentration decreased from the center of the leaf to the outer periphery. The midrib follows the same trend. Segments 1-6 of midvein contained the lowest concentrations of nitrosamines in the leaf. There was a marked increase of individual nitrosamines from segments 7-10, and these segments contained nitrosamine levels as high as those of the lamina on a microgram per gram basis. Lateral vein data showed approximately the same trend of having the lowest concentrations of TSNAs at the tip of the leaf.

To better present the distribution of TSNAs in the leaf, data for the lateral vein and lamina were combined using the weighted average of each segment. A surface graph for the total TSNA is presented in Figure 6 and gives a more complete view of the distribution of TSNAs in the tobacco leaf. The tip of the leaf contained the lowest concentration and the base of the leaf the highest level of TSNAs. Also, the TSNA concentration was lowest for the midrib in segments 1-6. In segment 8, the concentrations of TSNAs in lamina and midvein were equivalent. Along the length of the leaf, the TSNA concentration was highest in the leaf segments adjacent to the midvein. Higher concentration adjacent to the midvein may be the result of the slower dry down of the inner portion of the lamina during curing. Elevated moisture in cured lamina has been shown to result in higher concentrations of TSNAs (Burton et al., 1989a,b, 1990).

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The data from surface plot of Figure 6 also showed the apparent complexity of nitrosamine accumulation in tobacco and conflicting data from previous studies. Burton et al. (1989b, 1990) reported that the midvein of tobacco contained higher levels of TSNAs than lamina. These observations were in agreement with data from a study by Chamberlain et al. (1986). The above data were for samples taken from whole leaf. Conversely, data presented by Djordjevic et al. (1985, 1989) indicated that the midvein contains the lowest levels of nitrosamines. From the experimental data, their samples were a subsample taken from the center of the leaf (segments 3-7). Midvein in those samples would contain the lowest levels of TSNAs. If the TSNAs were calculated on a percent distribution, it would more clearly show that the sampling for TSNAs was not representative for the whole leaf. TSNA data based on percent of distribution in the leaf (Figure 7) show that the highest distribution is in the last three segments of the midvein. Sampling from middle segments of the leaf would result in low values for the TSNAs in the midvein which would not be indicative of the TSNA concentration in the whole leaf.

Relationships between Nitrite, Alkaloids, and TSNA Accumulation. Previous studies have indicated there is a relationship between alkaloid content and TSNA accumulation (Djordjevic et al., 1989). Also, there is a positive relationship between nitrite concentration and TSNA accumulation in the cured leaf (Burton et al., 1989a,b, 1990). Data from this indicate that nitrite content may be a better indicator of potential TSNA accumulation in the leaf than alkaloid concentration. From Figure 5, nornicotine concentration is greatest at the periphery of the leaf whereas nitrosonornicotine (NNN) concentration is highest along the segments adjacent to the midrib and at the base of the leaf. Except for the midrib, nitrite levels are generally uniform across the leaf, but nitrite increases from the tip to the base of the leaf. This increase of nitrite is also observed for the midrib. When nitrite data are presented as percent distribution in the leaf (Figure 8), its profile closely resembles the percent distribution of TSNAs (Figure 6). The TSNA distribution in leaf more closely parallels the nitrite levels than it does the alkaloid content of the leaf. Therefore, from this study, nitrite and not alkaloids or nitrate, appears to be the limiting factor for TSNA accumulation.

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Leaf Segment	Tissue	Weight g	NO_3^--N $\mu g\ g^{-1}$	NO_2^--N $\mu g\ g^{-1}$	Nicotine $mg\ g^{-1}$	Myosmine $mg\ g^{-1}$	Nornicotine $mg\ g^{-1}$	Anabasine $mg\ g^{-1}$	Anatabine $mg\ g^{-1}$	Total Alkaloids $mg\ g^{-1}$
7 C	L	10.9	540	1.42	66.40	0.19	0.60	0.117	0.68	68.00
7 D	L	2.8	510	1.55	75.06	0.18	0.71	0.122	0.79	76.86
8 A	L	9.7	1140	1.58	58.29	0.14	0.53	0.103	0.62	59.68
8 B	L	6.2	710	1.87	65.31	0.18	0.60	0.114	0.82	67.03
8 C	L	2.3	520	1.68	76.10	0.16	0.76	0.125	0.76	77.90
9 A	L	7.5	1340	1.79	46.86	0.14	0.47	0.094	0.63	48.19
9 B	L	0.7	-	-	-	-	-	-	-	-
10 A	L	1.7	1730	1.58	36.99	0.17	0.38	0.075	0.41	38.03
1	M	0.5	5450	1.19	13.81	0.06	0.20	ND	0.14	14.21
2	M	1.9	5450	1.19	13.81	0.06	0.20	ND	0.14	14.21
3	M	3.5	7150	1.14	10.29	ND	0.15	ND	0.10	10.53
4	M	6.0	8210	1.06	8.17	ND	0.12	ND	0.07	8.36
5	M	8.7	9250	1.12	7.64	ND	0.11	ND	0.07	7.81
6	M	10.4	8350	1.09	7.80	ND	0.11	ND	0.09	8.00
7	M	13.5	7720	1.25	6.73	ND	0.09	ND	0.07	6.89
8	M	16.4	7480	2.37	7.12	ND	0.08	ND	0.08	7.28
9	M	19.7	5430	3.05	5.63	0.04	0.06	ND	0.04	5.76
10	M	7.1	5430	3.05	5.08	0.04	0.07	ND	ND	5.19
2 A	V	1.4	960	1.37	24.61	0.13	0.31	0.042	0.25	25.34
3 A	V	1.9	1950	1.56	20.14	0.06	0.26	0.034	0.23	20.72
3 B	V	1.1	-	-	-	-	-	-	-	-
4 A	V	2.7	2970	1.74	17.05	0.04	0.23	ND	0.17	17.49
4 B	V	2.0	1440	1.85	23.75	0.08	0.30	0.041	0.30	24.47

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Table I. Continued (Page 3 of 3)

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Leaf Segment	Tissue	Weight g	NO_3^--N $\mu g\ g^{-1}$	NO_2^--N $\mu g\ g^{-1}$	Nicotine $mg\ g^{-1}$	Myosmine $mg\ g^{-1}$	Nornicotine $mg\ g^{-1}$	Anabasine $mg\ g^{-1}$	Anatabine $mg\ g^{-1}$	Total Alkaloids $mg\ g^{-1}$
4 C	V	0.6	-	-	-	-	-	-	-	-
5 A	V	3.2	3650	1.40	16.58	ND	0.23	ND	0.17	16.98
5 B	V	2.7	1980	1.58	26.04	0.07	0.31	0.044	0.25	26.71
5 C	V	1.2	-	-	28.68	0.19	0.37	0.058	0.31	29.60
6 A	V	4.0	4580	1.14	14.75	ND	0.23	ND	0.17	15.15
6 B	V	2.6	2530	1.82	29.42	0.06	0.35	0.050	0.29	30.17
6 C&D	V	1.2	-	-	27.75	0.15	0.39	0.058	0.27	28.61
7 A	V	3.3	4590	1.89	14.77	0.22	0.58	0.146	0.63	16.35
7 B	V	1.9	2530	2.10	21.67	0.31	0.83	0.201	0.91	23.93
7 C&D	V	0.6	-	-	-	-	-	-	-	-
8 A	V	2.0	3500	2.19	16.51	0.24	0.59	0.097	0.23	17.66
8 B&C	V	0.7	-	-	-	-	-	-	-	-
9 A&B	V	0.5	-	-	-	-	-	-	-	-

L = Lamina   ND = Not Detected

M = Midvein   "-" = Insufficient Sample for Analysis

V = Lateral Vein

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Table I. Distribution of Nitrate, Nitrite and Alkaloids in Leaf. (Page 1 of 3)

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Leaf Segment	Tissue	Weight g	NO_3^--N µg g^{-1}	NO_2^--N µg g^{-1}	Nicotine mg g^{-1}	Myosmine mg g^{-1}	Nornicotine mg g^{-1}	Anabasine mg g^{-1}	Anatabine mg g^{-1}	Total Alkaloids mg g^{-1}
1 A	L	10.1	380	1.58	33.06	0.37	0.37	0.078	0.49	34.35
1 B	L	0.5	-	-	-	-	-	-	-	-
2 A	L	12.4	730	4.46	35.99	0.16	0.36	0.066	0.44	37.02
2 B	L	10.0	230	1.29	45.42	0.22	0.42	0.080	0.57	46.71
2 C	L	1.4	220	1.34	48.82	0.29	0.49	0.095	0.61	50.30
3 A	L	11.7	1070	1.65	42.96	0.13	0.46	0.081	0.52	44.15
3 B	L	13.3	290	1.56	44.28	0.20	0.44	0.083	0.50	45.50
3 C	L	8.2	190	1.53	50.36	0.16	0.43	0.089	0.56	51.59
3 D	L	0.7	-	-	-	-	-	-	-	-
4 A	L	11.6	1500	1.80	47.25	0.12	0.47	0.087	0.52	48.45
4 B	L	13.7	520	1.66	47.26	0.14	0.43	0.089	0.51	48.42
4 C	L	12.9	310	1.67	54.56	0.15	0.48	0.093	0.59	55.87
4 D	L	3.4	250	1.55	66.91	0.20	0.55	0.108	0.66	68.43
5 A	L	11.1	1610	1.84	52.69	0.11	0.48	0.090	0.56	53.93
5 B	L	13.4	650	1.90	54.02	0.14	0.47	0.094	0.57	55.29
5 C	L	12.5	410	1.69	56.67	0.15	0.49	0.101	0.63	58.04
5 D	L	5.8	470	1.61	67.19	0.18	0.54	0.109	0.75	68.77
6 A	L	10.9	2340	1.50	57.01	0.12	0.53	0.100	0.70	58.45
6 B	L	11.9	650	1.12	57.40	0.22	0.53	0.109	0.65	58.90
6 C	L	12.5	490	1.20	63.14	0.24	0.52	0.108	0.65	64.66
6 D	L	5.6	420	1.30	68.74	0.17	0.59	0.109	0.68	70.28
7 A	L	10.0	1570	1.51	57.23	0.12	0.59	0.108	0.68	58.73
7 B	L	10.0	670	1.40	66.80	0.20	0.60	0.122	0.73	68.45

Table II. Distribution of Nitrosamines in Leaf. (Page 1 of 3)

<div align="right">16</div>

Leaf Segment	Tissue	NNN $\mu g\ g^{-1}$	NAT $\mu g\ g^{-1}$	NNK $\mu g\ g^{-1}$	TSNA $\mu g\ g^{-1}$
1 A	L	0.313	0.509	0.028	0.850
1 B	L	0.302	0.484	0.010	0.796
2 A	L	0.279	0.479	0.007	0.765
2 B	L	0.249	0.388	0.012	0.649
2 C	L	0.340	0.547	0.022	0.909
3 A	L	0.390	0.692	0.011	1.093
3 B	L	0.241	0.484	0.010	0.735
3 C	L	0.206	0.374	0.013	0.593
3 D	L	0.246	0.434	0.003	0.683
4 A	L	0.532	1.102	0.043	1.677
4 B	L	0.363	0.696	0.018	1.077
4 C	L	0.380	0.757	0.044	1.159
4 D	L	0.358	0.612	0.026	0.996
5 A	L	0.592	1.271	0.018	1.881
5 B	L	0.476	0.967	0.018	1.461
5 C	L	0.387	0.695	0.026	1.108
5 D	L	0.342	0.617	0.024	0.982
6 A	L	0.519	1.066	0.013	1.598
6 B	L	0.545	1.171	0.016	1.733
6 C	L	0.490	0.890	0.019	1.400
6 D	L	0.507	1.033	0.024	1.563
7 A	L	0.567	1.272	0.020	1.859

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Table II. Continued. (Page 2 of 3)

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Leaf

Segment	Tissue	NNN $\mu g\ g^{-1}$	NAT $\mu g\ g^{-1}$	NNK $\mu g\ g^{-1}$	TSNA $\mu g\ g^{-1}$
7 B	L	0.526	1.044	0.020	1.590
7 C	L	0.413	0.843	0.033	1.289
7 D	L	0.530	0.979	0.014	1.523
8 A	L	0.506	1.178	0.024	1.708
8 B	L	0.509	1.071	0.019	1.598
8 C	L	0.488	0.917	0.011	1.416
9 A	L	0.675	1.616	0.025	2.316
9 B	L	0.468	0.925	0.016	1.410
10 A	L	0.681	1.331	0.041	2.053
1	M	0.288	0.301	0.005	0.591
2	M	0.288	0.301	0.005	0.591
3	M	0.182	0.204	0.008	0.394
4	M	0.107	0.146	0.004	0.255
5	M	0.142	0.117	ND	0.259
6	M	0.164	0.299	0.008	0.466
7	M	0.375	0.831	0.006	1.212
8	M	0.531	1.087	0.014	1.632
9	M	0.654	1.130	0.036	1.821
10	M	0.770	0.592	0.069	1.431
2 A	V	0.457	0.558	0.180	1.196
3 A	V	0.353	0.294	0.018	0.664
3 B	V	0.281	0.342	0.028	0.651
4 A	V	0.304	0.431	0.020	0.754

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Table II. Continued. (Page 3 of 3)

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Leaf Segment	Tissue	NNN µg g⁻¹	NAT µg g⁻¹	NNK µg g⁻¹	TSNA µg g⁻¹
4 B	V	0.299	0.299	0.103	0.702
4 C	V	0.383	0.478	0.028	0.890
5 A	V	0.297	0.460	0.015	0.771
5 B	V	0.371	0.474	0.015	0.861
5 C	V	0.621	0.846	0.115	1.582
6 A	V	0.465	0.541	0.022	1.029
6 B	V	0.542	0.637	0.043	1.222
6 C&D	V	0.409	0.439	0.022	0.870
7 A	V	0.407	0.464	0.042	0.913
7 B	V	0.422	0.533	0.034	0.989
7 C&D	V	0.830	0.908	0.129	1.867
8 A	V	0.501	0.726	0.014	1.241
8 B&C	V	0.654	0.623	0.027	1.304
9 A&B	V	0.531	0.791	0.034	1.356

* All values µg/g

ND = Not Detected

L = Lamina

M = Midvein

V = Lateral Vein

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LIST OF FIGURES

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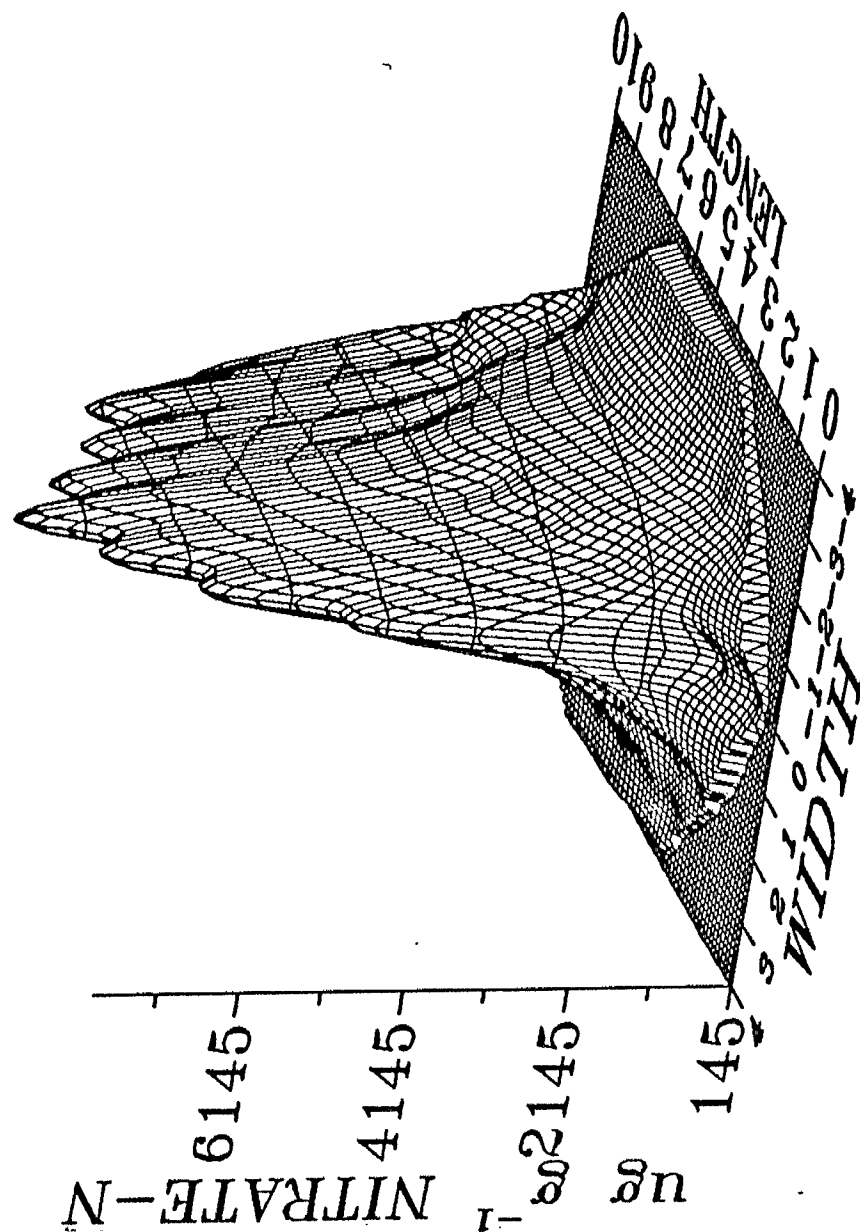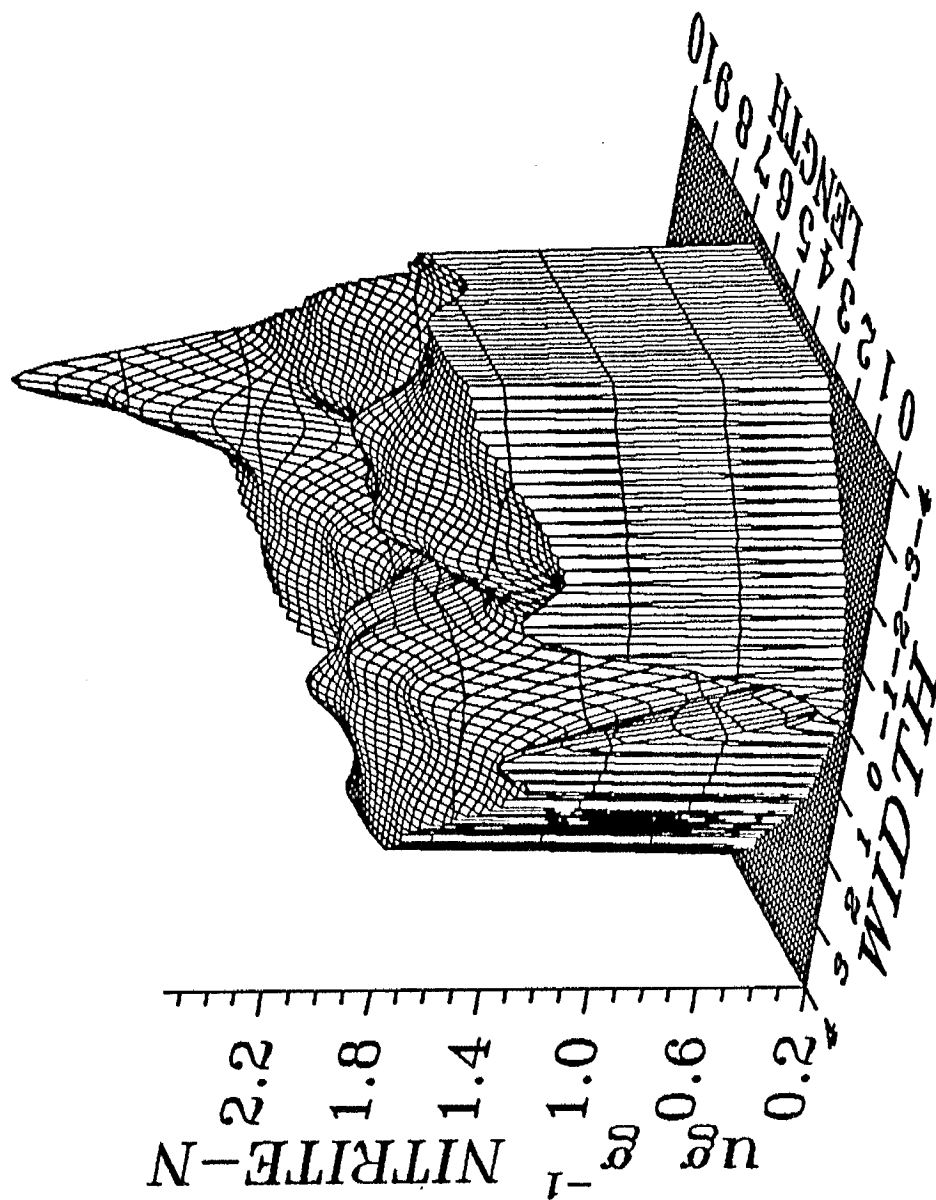

Figure 1

Figure 2

Figure 3
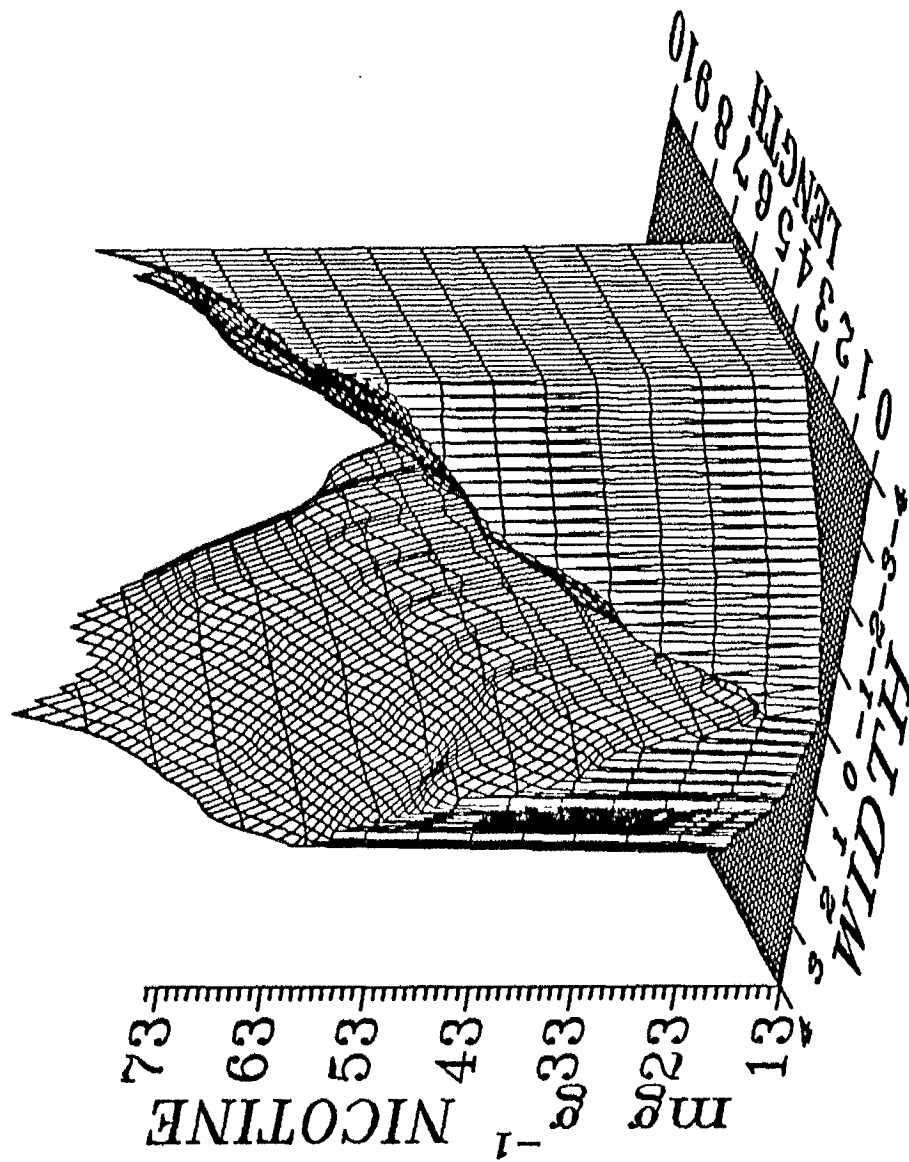
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Figure 4

Figure 5

Figure 6

PERCENT DISTRIBUTION TSNA

Figure 7

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Figure 8

PERCENT DISTRIBUTION NITRITE-N
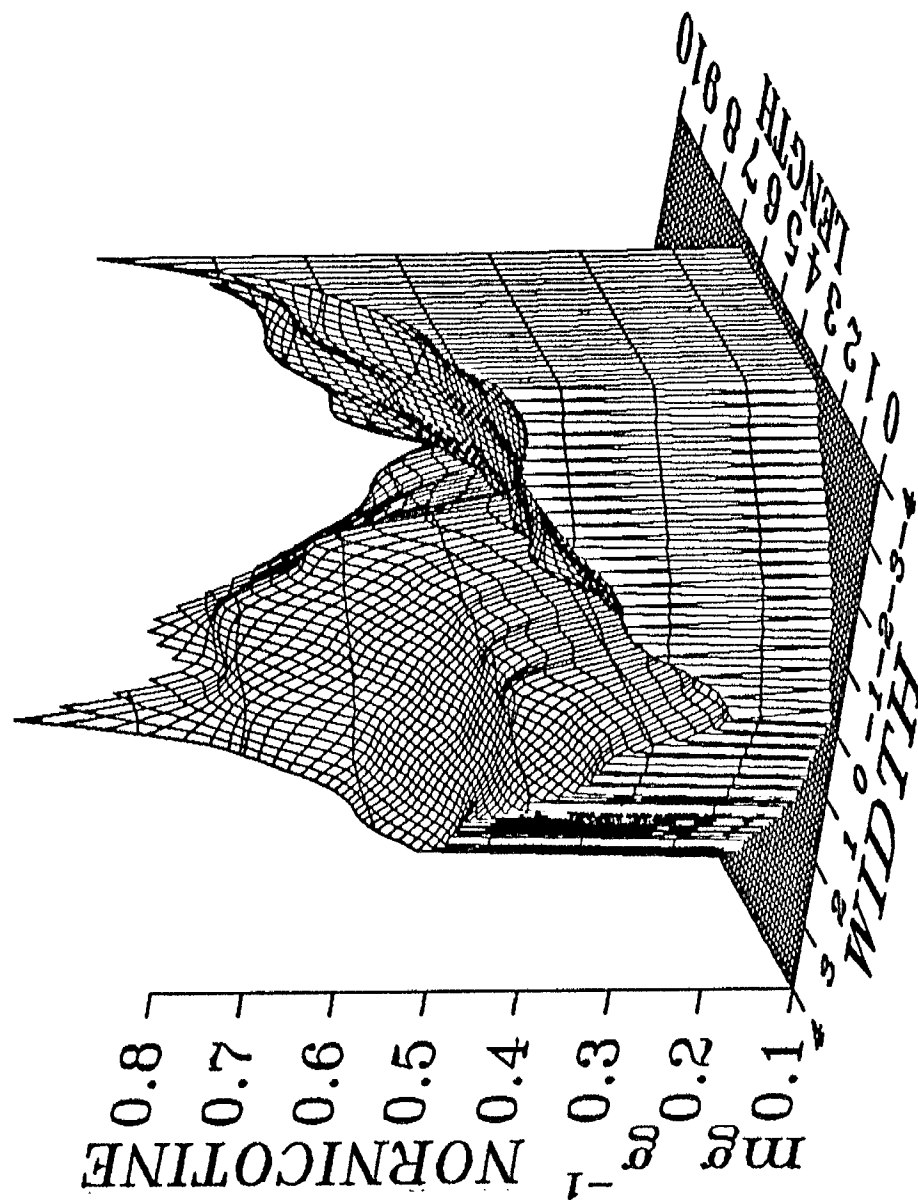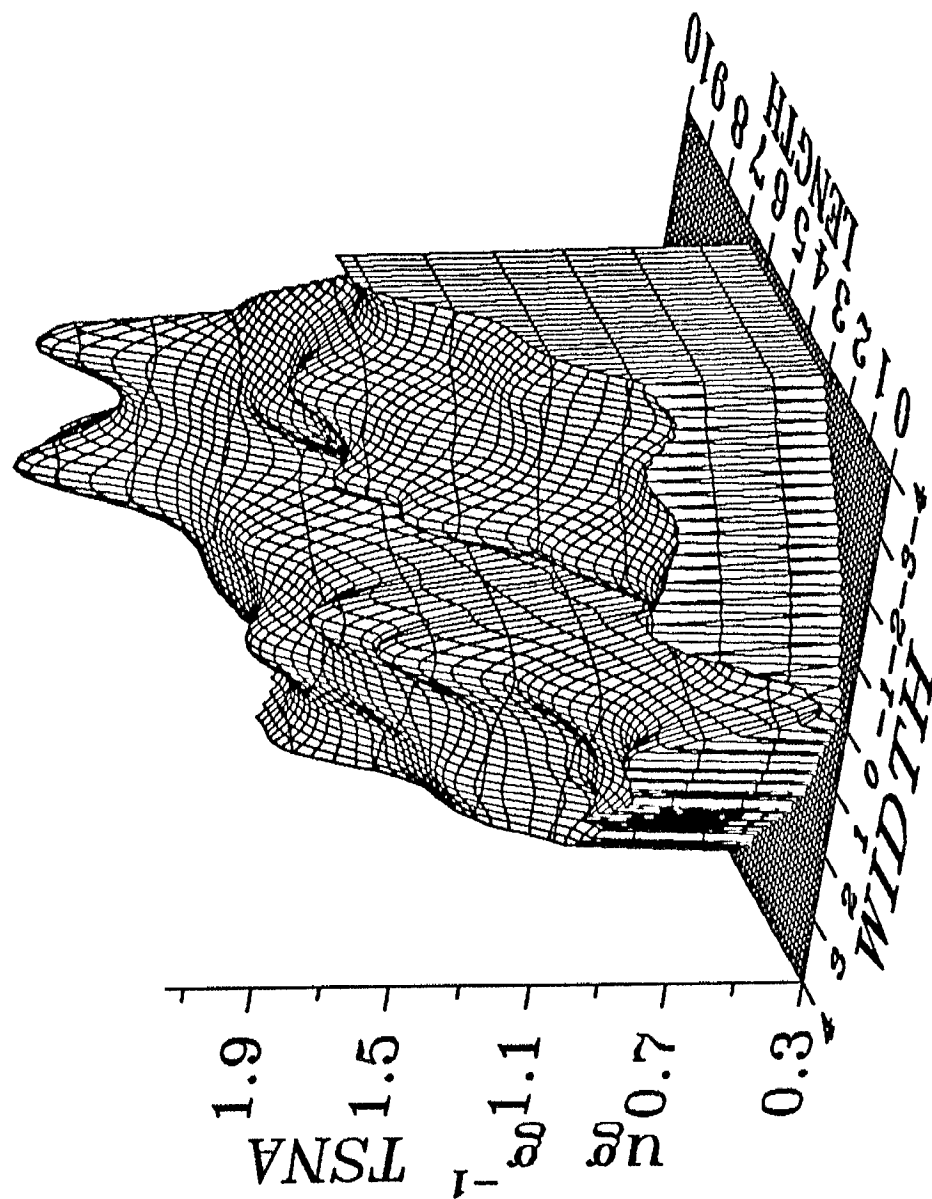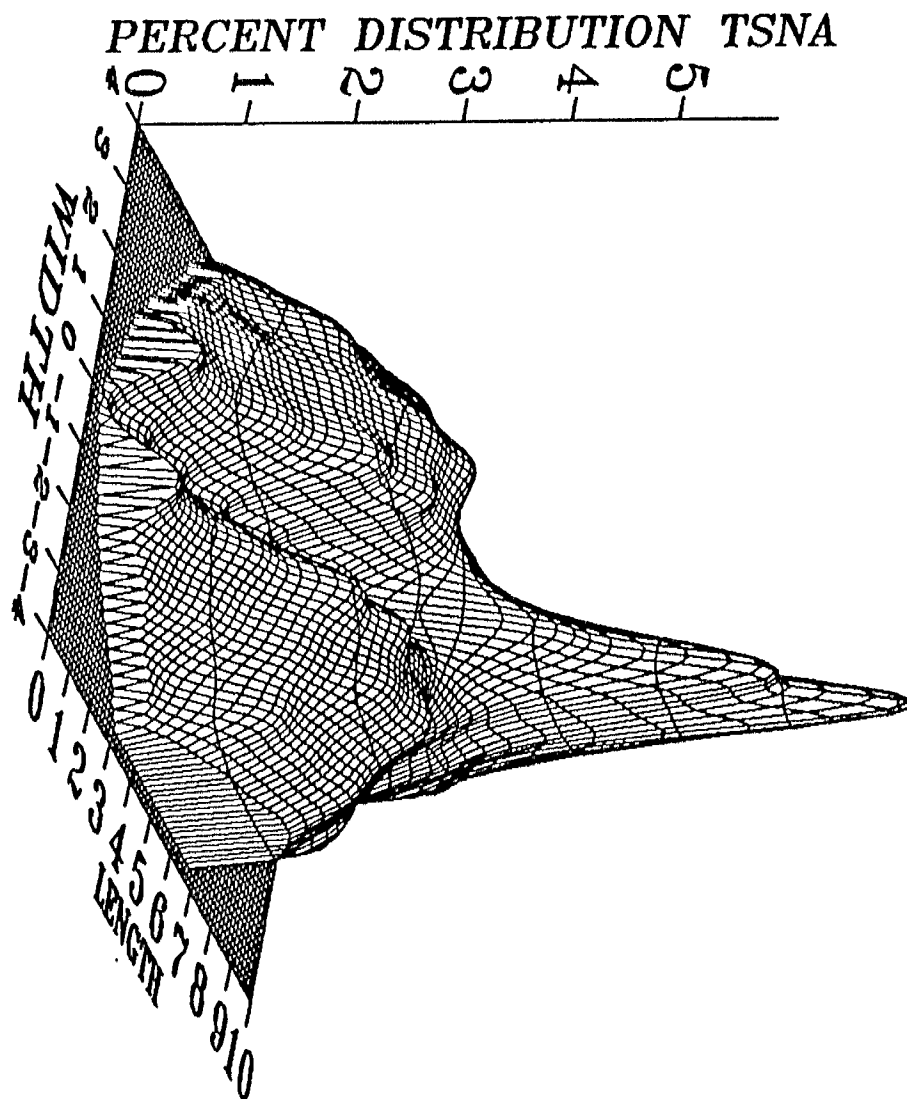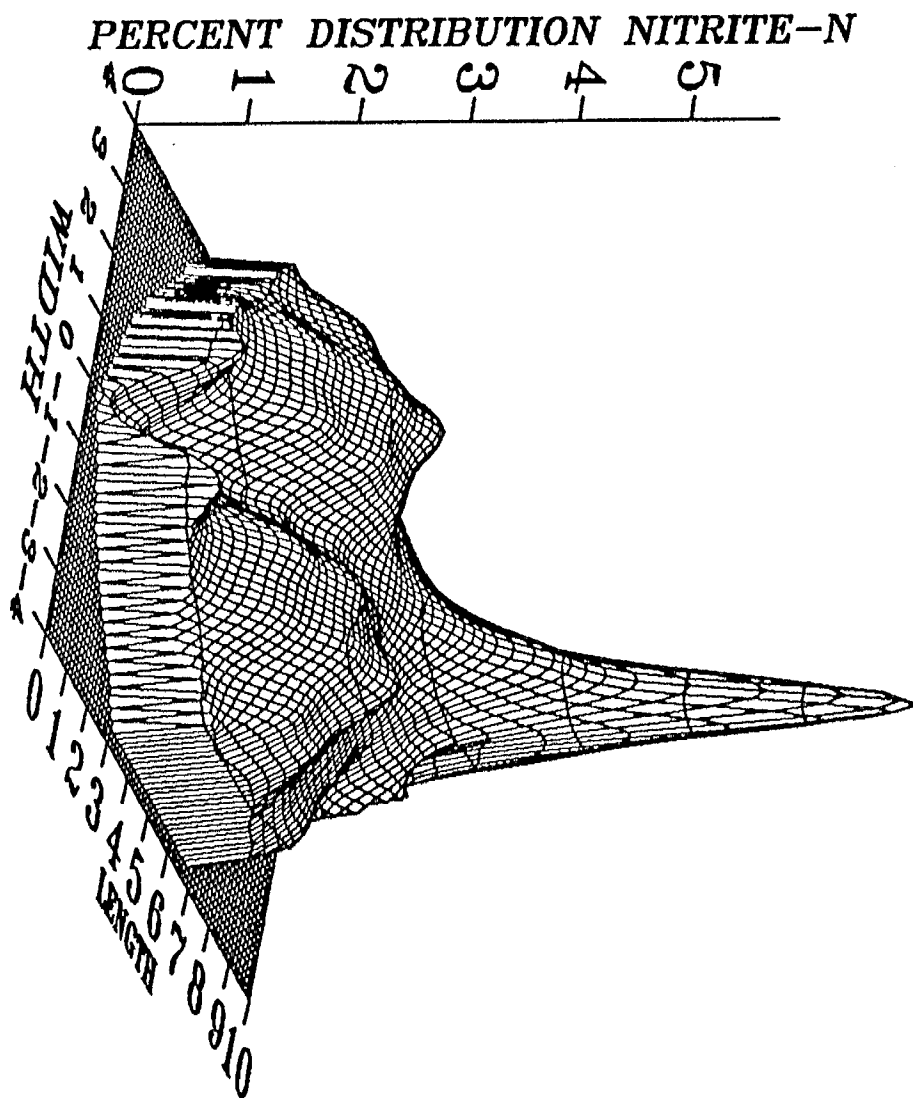
Effects of Storage Conditions on Levels of Tobacco-Specific N-Nitrosamines and
N-Nitrosamino Acids in U.S. Moist Snuff

The consumption of oral snuff in the USA has steadily risen from 43.8 million
lbs in 1982 to 56.0 million lbs in 1992 (U.S. Department of Agriculture 1992). One
major reason for this trend is the growing prevalence of snuff dipping among male
adolescents (Orlandi and Boyd, 1989); constraint of smoking in public places may be
another reason. Smokeless tobacco is known to contain at least 28 carcinogens
including the tobacco-specific nitrosamines (TSNA, Hoffmann et al., 1992). The latter
are known carcinogens in animals and are regarded as at least partly responsible for
the excess of oral cancer among snuff-dippers (Preston-Martin, 1991). During the past
decade, the levels of N-nitrosamines in the leading snuff brands in the USA and
Sweden have gradually declined (Djordjevic et al., 1993; Brunnemann and Hoffmann,
1991), even though a new snuff brand, introduced in the USA in 1990 and sold until
1992, contained the highest concentrations of TSNA (up to 177 + 95 ppm) and
N-nitrosamino acids (NAA, up to 85 ± 58 ppm) ever determined in a commercial
tobacco product (Djordjevic et al., 1993). Andersen et al. (1989, 1991) have recently
shown that nitrite and TSNA levels increased substantially in the University of
Kentucky reference moist snuff when the product was stored at 24°C and 32°C,
respectively, for 52 weeks, in sealed 1-liter Mason jars, or in the original tins that were
exposed to ambient air. The increase of the highly carcinogenic
4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosonornicotine
(NNN), was 14- and 33-fold, respectively. Higher moisture content had a greater
effect than higher temperature on the increases of TSNA and nitrite levels in the snuff.
NNK concentration in a commercial moist snuff exposed to air in a porous paper bag
doubled in just two weeks (Hoffmann and Adams, 1981). When burley tobacco
(approximately 10% in moist snuff) was exposed to adverse temperature and humidity
conditions (32°C; 90% RH), significant increases in NNN, NNK, and nitrite levels
occurred within 7 days but then the levels of these constituents declined (Burton et al.,
1989). Treatment of tobacco with streptomycin or rifampicin did not inhibit the

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formation of nitrite or nitrosamines. When air-cured or fire-cured KY 171 dark tobaccos, which are also utilized for moist snuff formulations (approximately 25%), were stored at 24°C and 32°C for 52 weeks, no significant changes in TSNA levels were observed (Andersen et al., 1990). In view of these findings with reference snuff and raw tobaccos, and because of the rising consumption of moist snuff and the carcinogenicity of several TSNA and NAA, we deemed it important to undertake a systematic study of the effect of storage conditions on N-nitrosamine formation in a leading commercial U.S. snuff brand.

In this study we also identified and quantified the NNK-derived 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) in moist snuff. Like NNK, NNAL is a strong inducer of tumors in laboratory animals (Hoffmann et al., 1993) and its presence in moist snuff adds to the overall carcinogenic potential of smokeless tobacco.

Experimental Procedures

Materials. A leading commercial U.S. moist snuff brand was purchased from retailers in Westchester County, NY, in 1991 and 1992. The 1991 snuff was manufactured on June 27th, 1991, purchased on July 2nd and stored immediately in a cold room. The first analysis of this sample was done on July 9th, 1991, i.e. within 2 weeks of the manufacture date. Another 1991 snuff sample (batch #2) was manufactured on August 15th, purchased on August 20th, and first analyzed on August 21st. The 1992 snuff was manufactured on September 24, 1992, purchased on October 6th, and the first analysis was performed on October 8th, 1992. The experimental design was as follows: (A) snuff was stored in a cold room (4°C); (B) snuff was stored on the shelf at ambient temperature and humidity, and (C) snuff was kept in an incubator at 37°C, at 85% relative humidity (RH), with an air flow of 20 mL/min. The both batches of the 1991 snuff were stored in individually sealed cardboard tins, whereas 1992 snuff was stored in plastic-wrapped 10-tin sleeves. Samples to be analyzed were taken at various time points, up to 32 weeks from the beginning of the experiment. At each time point a set of 3-5 individually sealed tins, or one 10-tin

sleeve was opened and the tobacco was frozen in liquid nitrogen, freeze-dried, and ground.

For the identification of NNAL, a newly introduced commercial snuff brand containing high levels of carcinogenic TSNA (65 ppm NNN and 4.3 ppm NNK, Djordjevic et al., 1993) was utilized. This brand had been purchased in Reno, NV, in 1991.

Reagents and Standards. All chemicals and solvents were analytical reagents of the highest purity from J.T. Baker Chemical Co., Phillipsburg, NJ, Fischer Scientific Co., Fair Lawn, NJ, Aldrich Chemical Company Inc. Milwaukee, WI, and Alltech Associates, Inc., Deerfield, IL. Individual TSNA and NAA, which were utilized as standards for the analyses, were obtained as follows: NNN and NNK were synthesized according to Hu et al. (1974) and Hecht et al. (1977); NNAL was synthesized by reduction of NNK with sodium borohydride (Hecht et al., 1980); N'-nitrosoanatabine (NAT), N-nitrososarcosine (NSAR), and N-nitrosoproline (NPRO) were purchased from the NCI Chemical Carcinogen Reference Standard Repositories, c/o Midwest Research Institute, Kansas City, MO. 3-(Methylnitrosamino)propionic acid (MNPA) and 4-(methylnitrosamino)butyric acid (MNBA) were synthesized according to Gerjovich and Harrison (1966), and N-nitrosoguvacoline (NG) according to Christensen and Krogsgaard-Larsen (1977). The purity of the reference compounds (>99%) was verified by capillary GC with flame ionization detection, and by NMR and MS. The reference mixture of seven volatile N-nitrosamines (VNA), as well as Nnitrosodipropylamine (NDPA) were purchased from Thermedics, Woburn, MA.

Apparatus. The instruments used in this study were previously described (Djordjevic et al., 1989; 1991; Brunnemann et al., 1982). Snuff was stored at "high temperature/high humidity" conditions (37°C, 85% RH) in an incubator of the Thermolyne Compact CO_2 Series 5000 (Fisher Scientific, Springfield, NY). The ambient temperature was monitored by a Dickson Self-Contained Seven-Day Temperature Recorder (7.2-32.2°C range; Dickson Company, Addison, IL).

GC-MS analysis of derivatized NNAL was carried out with a Hewlett-Packard GC Model 5890 interfaced with a Hewlett-Packard 5970 MSD in the electron impact

mode. The analyses were performed by splitless injection (purge time 1 min) on a 0.25 mm x 30-m OV225 (0.5 m film thickness) fused silica capillary column purchased from On-Site Instruments, Columbus, OH. The injector was kept at 230°C, the transfer line temperature was 250°C. Helium was the carrier gas (head pressure, 9 psi). The oven temperature was programmed as follows: 60°C for 2 min; then 30°C/min to 140°C; this was held for 3 min; then heated 4°C/min to 210°C and held for 120 min. Under these conditions, TMSi-NNAL eluted after 37.62 min.

Methods. The analytical methods for the determination of water, alkaloids, nitrite and nitrate-nitrogen, pH, tobacco-specific N-nitrosamines, and N-nitrosamino acids in snuff were previously reported (von Bethmann et al., 1961; Armstrong et al., 1967; Crutchfield and Burton, 1989; Djordjevic et al., 1989, 1991). Volatile N-nitrosamines (VNA) were analyzed by means of a procedure that was slightly modified from that described by Brunnemann et al., 1977, 1982): 10 g of snuff were extracted for 2 h under magnetic stirring with 100 mL citrate buffer (pH 4.5) containing 20 mM ascorbic acid and 5 g/mL NDPA as an internal standard (dichloromethane used for the partitioning of the aqueous tobacco extract was free of VNA). The concentrations of individual VNA were calculated on the basis of their recoveries which were determined in separate experiments in which 1 mL of a reference standard mixture was carried through the same analytical procedure as the tobacco samples. The recoveries of the seven VNA ranged from 46 % (N-nitrosodimethylamine, NDMA) to 80% (N-nitrosodibutylamine, NDBA) with coefficients of variations ranging from 9.4% for NDBA to 35% for NDMA (average of 4 experiments). The detection limit of each VNA was 50 pg/injection. All samples were both extracted and analyzed by GC-TEA in duplicates.

NNAL and other TSNA were extracted from moist snuff by partitioning an aqueous extract of the tobacco at pH 9 with ethyl acetate (Djordjevic et al. 1989). Upon removal of ethyl acetate by evaporation in vacuum, the extract was derivatized with bistrimethylsilyltrifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMC) at 70°C for 15 min prior to analysis by GC-TEA, GC-FID, and GC-MSD.

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Results and Discussion

Table I lists analytical data for tobacco-specific N-nitrosamines, N-nitrosamino acids and related parameters in a leading U.S. moist snuff brand, that had been stored in a cold room at 4°C. During 32 weeks of storage no significant change in the levels of any of the measured components was observed except for N-nitrosoproline (NPRO) which had decreased significantly ($p < 0.001$). The coefficients of variation ranged from 2% for pH to about 15% for nitrate and nitrite, NNK, MNPA and MNBA. Moist snuff purchased in 1992 contained lesser amounts of both TSNA and NAA by comparison to the same type of snuff purchased in 1991, a trend which we reported earlier (Djordjevic et al., 1993).

The chemical composition of snuff changed significantly during storage at ambient room temperature (Table II). When individually sealed tins were kept at room temperature, range 13-27°C, the moisture content of the snuff was reduced by more than 50% within 24 weeks, probably due to evaporation. Nicotine content varied somewhat during storage, pH decreased, while levels of nitrite, individual TSNA, and NAA in tobacco started to rise after about 4 weeks of storage. These changes were even more pronounced after 24 weeks of storage (NNN and MNPA levels increased 2.9-fold; NNK and MNBA levels increased 4.4 and 6.3 times, respectively). This is the first study that reports on NAA levels in tobacco during storage.

When moist snuff was shelved in plastic-wrapped 10-tin sleeves for 8 weeks at room temperature (range 13-23°C), trends of increasing concentrations of TSNA, NAA, and nitrite were also observed; surprisingly, in this case moisture appeared to accumulate in tobacco over time.

When moist snuff was stored in an incubator (37°C; 85% RH; 20 mL/min air flow), moisture, pH, nitrite, TSNA, and NAA, increased throughout the storage period (Table III). Changes in nicotine levels were not significant. During 8 weeks, the individual TSNA increased up to 4.7-fold (NNN and NAT rose 2.5-fold; NNK 4.7-fold). The greatest increase, both at ambient temperature and at 37°C, occurred for NNK, one of the most potent carcinogens in smokeless tobacco products (Hoffmann et al., 1993).

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During storage, not only the levels but also the composition of the VNA in snuff changed (Table IV). At the beginning of the study, snuff contained only NDMA and N-nitrosopyrrolidine (NPYR; 7.4 ppb and 15.3 ppb, respectively). During 8 weeks of storage in a cold room, the levels of these two VNA did not change significantly. However, two additional VNA were detected, namely N-nitrosopiperidine (NPIP) and N-nitrosomorpholine (NMOR; 8.2 and 2.8 ppm, respectively). The occurrence of the latter was probably due to migration of morpholine from the waxed cardboard container into the snuff where it was subsequently nitrosated. This phenomenon had been reported by us earlier (Brunnemann et al., 1982). After 8 weeks of storage, at ambient temperature or at 37°C, the levels of all VNA had increased significantly. The concentrations of the highly carcinogenic NDMA and NPYR had at this point increased 5-fold. The carcinogens NDBA, NPIP, and NMOR, which were not originally detected in moist snuff, by now amounted to 66.1, 8.9 and 19.7 ppb, respectively. Although the levels of VNA in tobacco are 2-3 orders of magnitude lower than those of TSNA and NAA, the latter finding is important in view of the high carcinogenic potency of these compounds (Hoffmann et al., 1993; Preussmann and Stewart, 1984). In addition to known VNA, two unknown volatile compounds formed during storage were observed in the GC-TEA chromatogram (peaks #1 and #2, Figure 1). When the VNA extracts containing unknown peaks were exposed to UV radiation (365 nm; Krull et al., 1979) for one hour, the peak #1 disappeared while the peak #2 remained in the GC-TEA chromatogram, suggesting that only #1 is a N-nitroso compound. The VNA extracts that contained peaks of unknowns were also treated with 10% hydrobromic acid in glacial acetic acid. Again, only #1 vanished while #2 remained in the chromatogram, though its intensity was somewhat reduced, suggesting that this unknown is possibly an organic nitro compound ($N-NO_2$, $O-NO_2$, or $C-NO_2$, Krull et al., 1979).

It is evident from this study that the levels of the highly carcinogenic tobacco-specific N'-nitrosamines and volatile N-nitrosamines in commercial moist snuff can rise significantly upon storage at room temperature over extended periods (> 4 weeks). Because it has been shown that morpholine from package material can diffuse

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into the snuff where it forms NMOR (Brunnemann et al., 1982), additional studies on packaging materials and product quality are indicated. Certainly, the nature of packaging materials can influence product composition as was earlier shown in this study. In addition, there is sufficient evidence to conclude that factors other than storage conditions, such as blending of the tobacco and manufacturing technology, contribute to extremely high levels of TSNA and NAA. A primary example of this is seen in the chemical analysis of a commercial moist snuff that had been introduced on the U.S. market in 1990 (Djordjevic et al., 1993).

Earlier, NNAL had been only tentatively identified in moist snuff (Brunnemann et al., 1987). Since the level of NNAL in moist snuff was about twice that of NNK in a sample purchased in Nevada (9.6 ppm vs. 4.3 ppm), this sample afforded us the opportunity to positively identify the structure of this TSNA. Solvent partition of the aqueous tobacco extract at pH 9 and subsequent silylation with BSTFA + 1% TMC yielded the compound which eluted at the same time as reference NNAL-TMSi when analyzed by GC-TEA, GC-FID, and GC-MSD. Also, the mass spectra of the reference compound and of NNAL-TMSi isolated from moist snuff were identical (Figure 2).

The concentration of NNAL in a leading U.S. moist snuff brand did not increase significantly during 8 weeks storage at ambient temperature but it doubled during the storage at 37°C (from 0.29 to 0.65 ppm).

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Table I. Changes in the Chemical Composition of a Moist Snuff Brand (U.S.A.)During Storage*

A. Cold Room (4°C)

Storage Duration (weeks)	H₂O %	pH	NO₂-N mg/g	NO₃-N mg/g	Nicotine mg/g	TSNA			NAA		
						NNN	NNK	NAT	MNPA	MNBA	NPRO
						μg/g			μg/g		
Individual Tins (1991)											
0	56.7	6.69	0.084	4.17	18.6	7.99	0.77	5.64	4.45	0.41	8.09
2	58.0	6.80	0.080	3.94	19.4	8.11	0.82	5.38	4.23	0.42	8.32
4	57.6	6.95	0.066	3.53	16.7	7.09	0.64	4.69	4.37	0.39	7.13
8	59.8	7.09	0.060	3.77	18.1	7.40	0.68	5.27	4.69	0.40	8.24
11	59.3	6.99	0.083	3.93	16.8	7.31	0.58	4.68	3.54	0.36	6.18
16	61.8	6.82	0.087	3.79	19.5	6.12	0.52	3.78	3.42	0.37	5.92
26	56.3	6.83	0.086	4.82	17.4	7.05	0.64	4.58	2.96	0.28	5.05
32	55.6	7.02	0.064	4.72	16.6	6.60	0.77	4.59	3.37	0.51	3.66
AVG	58.1	6.90	0.076	4.08	17.9	7.21	0.68	4.83	3.88	0.39	**
STD	1.9	0.13	0.011	0.43	1.1	0.62	0.10	0.55	0.59	0.06	
10-Tin Sleeves, Wrapped in Plastic (1992)											
0	48.5	6.96	0.058	5.68	16.7	5.64	0.60	3.40	2.84	0.29	4.50
8	49.9	7.12	0.088	5.47	16.3	5.85	0.54	3.27	2.96	0.24	4.86

* Values are based on dry weight.

** Significant decreasing trend over time (P<0.001).

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Table II. Changes in the Chemical Composition of a Moist Snuff Brand (U.S.A.) During Storage*

B. Laboratory Shelf (Ambient Conditions)

Storage Duration (weeks)	H₂O %	pH	NO₂-N mg/g	NO₃-N mg/g	Nicotine mg/g	TSNA NNN	TSNA NNK	TSNA NAT	NAA MNPA	NAA MNBA	NAA NPRO
						μg/g			μg/g		
Individual Tins (1991)											
(Batch #1)											
0	56.7	6.69	0.084	4.17	18.6	7.99	0.77	5.64	4.45	0.41	8.09
2	54.5	6.78	0.136	4.13	14.9	8.86	1.33	6.63	5.70	0.63	9.29
4	55.6	7.09	0.622	3.34	16.2	8.88	1.31	6.75	5.55	0.60	8.57
8	51.6	7.58	0.652	4.39	17.4	11.34	1.74	7.08	7.80	1.08	11.00
(Batch #2)											
0	56.7	6.91	0.113	4.88	20.6	4.76	0.54	3.13	2.81	0.26	4.28
12	42.5	6.84	0.469	3.71	13.7	6.64	0.80	3.52	4.39	0.68	5.56
24	21.7	6.85	0.270	3.70	13.1	13.70	2.39	9.74	8.08	1.64	15.56
10-Tin Sleeves, Wrapped in Plastic (1992)											
0	48.5	6.96	0.058	5.68	16.7	5.64	0.60	3.40	2.84	0.29	4.50
2	53.1	7.00	0.122	5.74	19.4	5.48	0.54	3.24	2.66	0.25	4.08
4	56.0	7.01	0.369	5.39	17.8	5.50	0.85	3.85	3.42	0.47	5.08
8	57.6	7.31	1.009	5.54	20.2	8.02	1.31	5.12	4.26	0.68	8.22

* Values are based on dry weight.

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Table III. Changes in the Chemical Composition of a Moist Snuff Brand (U.S.A.) During Storage*

C. Incubator (37°C; 85 % RH; 20 mL/min Air Flow)

Storage Duration (weeks)	H_2O %	pH	NO_2-N mg/g	NO_3-N mg/g	Nicotine mg/g	TSNA			NAA		
						NNN	NNK μg/g	NAT	MNPA	MNBA μg/g	NPRO
10-Tin Sleeves Wrapped in Plastic (1992)											
0	48.5	6.96	0.058	5.68	16.7	5.64	0.60	3.40	2.84	0.29	4.50
2	54.3	7.16	0.392	5.93	16.7	7.42	0.93	5.00	3.67	0.38	6.13
4	58.0	7.33	0.551	5.73	14.2	9.33	2.94	7.94	7.41	1.25	10.27
8	58.0	7.44	0.466	5.42	16.3	15.22	3.51	8.98	13.87	2.47	19.63

* Values are based on dry weight.

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Table IV. Changes in the Chemical Composition of the U.S. Moist Snuff Brand During Storage.
Volatile N-Nitrosamines*

Place	Storage Duration (weeks)	NDMA	NDBA	NPIP ng/g	NPYR	NMOR
10-Tin Sleeves, Wrapped in Plastic (1992)						
Cold Room	0	7.4	ND**	ND	15.3	ND
(4°C)	8	5.3	ND	8.2	17.6	2.8
Lab Shelf (13-23°C)	8	21.3	41.0	8.6	70.4	19.7
Incubator (37°C)	8	38.6	66.1	8.9	71.4	17.6

* Values are based on dry weight.
** ND, not detected.

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Legends to Figures

Figure 1. GC-TEA traces of volatile N-nitrosamines (VNA) in a commercial moist snuff brand stored at different temperatures for 8 weeks (#1 and #2 are unknown compounds).
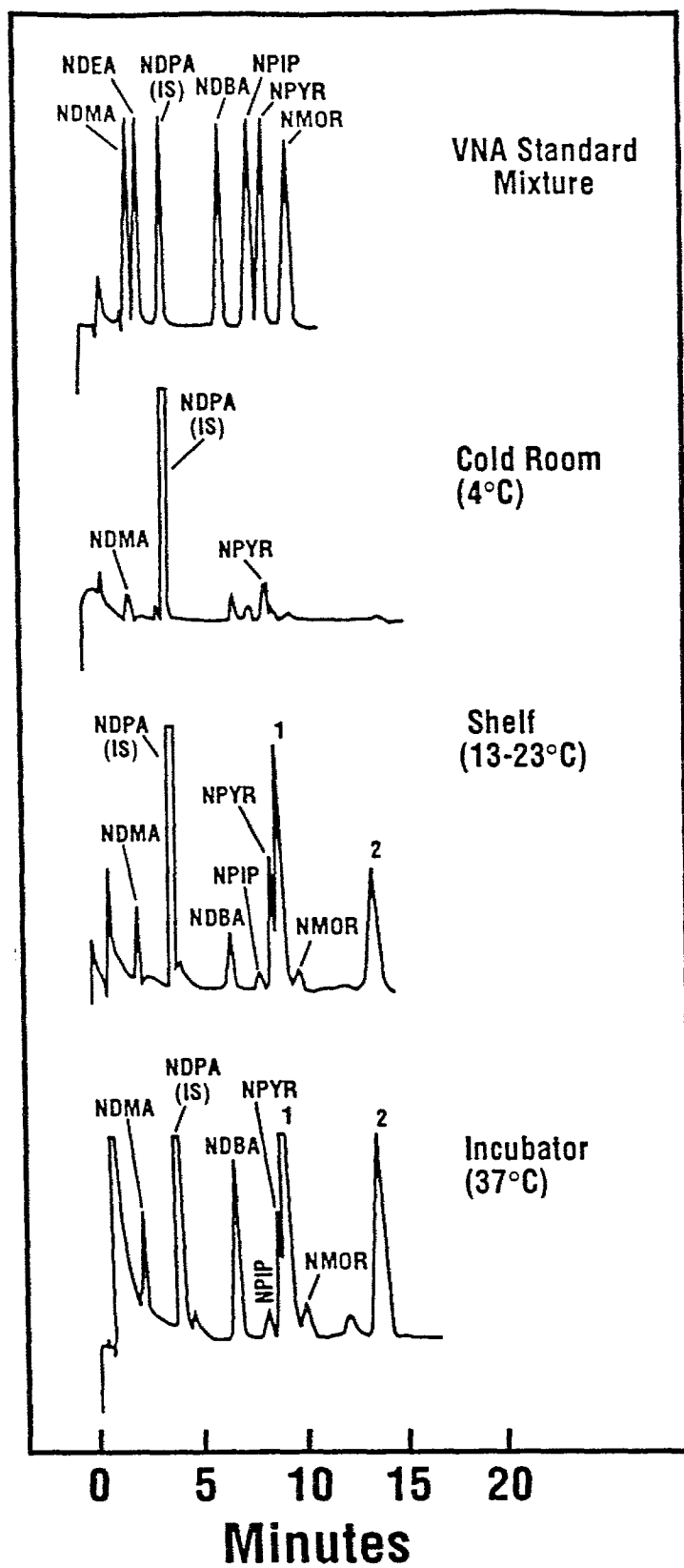
Figure 2. GC-MSD analysis (electron impact mode) of NNAL-TMSi: (a) total ionization chromatogram of pH 9 fraction from snuff extract, (b) mass spectrum of NNAL-TMSi from snuff, and (c) mass spectrum of reference NNAL-TMSi.
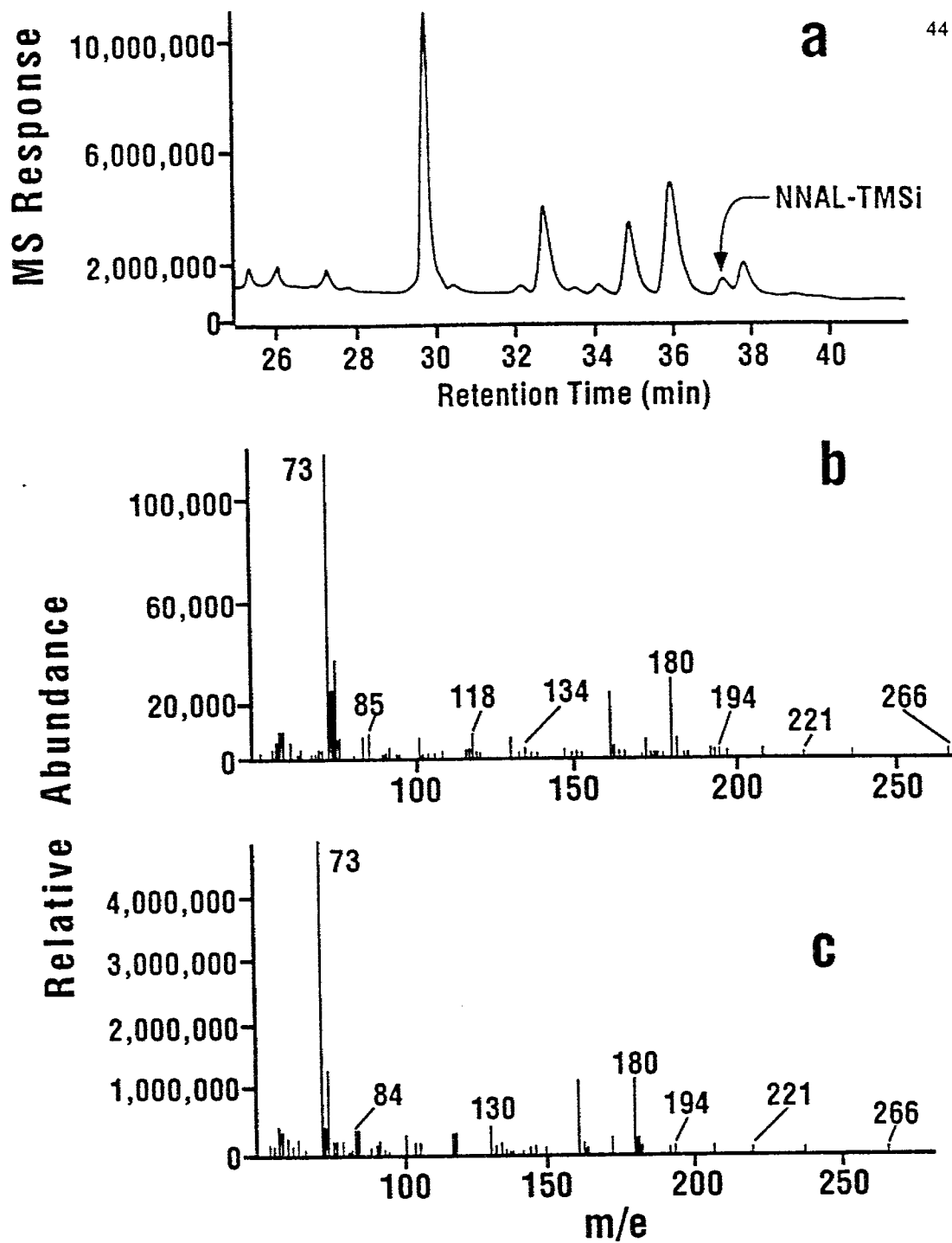
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Figure 1

TEA Response

NDEA   NDPA   NPIP
        (IS)   NDBA   NPYR
NDMA   NMOR
                                VNA Standard
                                Mixture

NDPA
(IS)
                                Cold Room
NDMA   NPYR   (4°C)

NDPA
(IS)   1
                                Shelf
NDMA   NPYR   (13-23°C)
          NPIP
        NDBA   2
                NMOR

        NDPA   NPYR
NDMA   (IS)   1   2
            NDBA
                                Incubator
                                (37°C)
            NPIP
                NMOR

0   5   10   15   20
Minutes

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Figure 2

Nicotine demethylation in *nicotiana*

N-demethylation of nicotine is the primary source of nornicotine biosynthesis in tobacco. Nornicotine is a minor alkaloid of commercial tobaccos but is the major alkaloid in several *Nicotiana* species (Saitoh et al., 1985). In mammals, demethylation of nicotine to nornicotine in the liver is apparently a minor pathway of nicotine metabolism which has been postulated to occur by the same P-450-dependent mechanism (C-hydroxylation) as the formation of cotinine, the major metabolite of nicotine in mammals (Kyerematen and Vesell, 1991; Jacob and Benowitz, 1991).

Early reports of nornicotine formation in mammals were equivocal and 'intact cell' models may be required to demonstrate nornicotine formation *in vitro* (Kyerematen and Vesell, 1991). Unsuccessful attempts of purification of a soluble nicotine demethylating enzyme from plants have been noted in the literature and this lack of success may indicate that the enzyme is microsomal like the P-450-dependent mammalian enzyme proposed for nornicotine formation.

Stereoisomers of nornicotine are behaviourally and physiologically active in mammalian systems and may contribute to the pharmacological action of tobacco (Risner et al., 1988). Also, in the plant nornicotine is readily acylated and nitrosated at the N' position to produce a series of N'-acylnornicotines and N'-nitrosonornicotine (NNN). NNN has carcinogenic activity (Hect et al., 1980) and both N'-acylnornicotines and NNN are undesirable constituents of tobacco. N'-acylnornicotines do have biological activity against selected insects (Severson et al., 1988). Elimination of nornicotine formation would eliminate the pharmacological action of nornicotine and its derivatives.

The objective of this study was to characterize *in vivo* nicotine demethylation in *Nicotiana* species.

Materials and Methods

Small plant tissue pieces (3 mg) were added to 1.5 mL glass vials and 2 μL of 2 mM (RS)-2'-^{14}C nicotine, pH 6, were added directly to tissue pieces and incubated at

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23-27°C. Nicotine solutions were adjusted to pH 6 without buffering. Root tissue pieces also were incubated with 10 μM or 20 μM tetcyclasis, a P-450 enzyme inhibitor. Alkaloids were extracted from tissue by adding 20 μL of a methanolic solution of 50 mM unlabelled nicotine and nornicotine and 5 μL of 2 M NaOH to each vial. Ten μL portions of extract were chromatographed on silica gel plates (with fluorescent indicator) using a solvent system of $CHCl_3:MeOH:aq\ NH_3$ (85:15:2). Nicotine and nornicotine areas were removed and ^{14}C determined.

Susceptibility of root cortex (a non-chlorophyllus tissue) to carbon monoxide was tested in the presence of a mixture of 80% CO/20% O_2 added immediately before incubation of the tissue with 10 μL of 2 mM $2'$-^{14}C nicotine in darkness or with white light (1,300-1,500 μE m^{-2} s^{-1}).

Hydroponically grown *N. glauca* plants were root fed 2 days with 2 mM phenobarbital and lamina, petiole and roots were tested for induction of nicotine demethylation activity. Also, juvenile leaves of *N. sylvestris* and *N. tabacum* were exposed to the growth regulator ethylene in an attempt to induce nicotine demethylation to nornicotine.

Results and Discussion

Initial studies of nicotine demethylation were carried out by feeding nicotine through the cut petioles of detached leaves of *N. glauca*, an anabasine accumulator, and *N. africana*, a nicotine/nornicotine accumulator. Nicotine levels in midrib and lamina declined rapidly in the first two days after feeding, while nornicotine concentrations rapidly increased. Essentially all of the nicotine fed was converted to nornicotine by five days after feeding. *N. glauca* has the competency to demethylate nicotine to nornicotine just as the nicotine/nornicotine-accumulating species do.

In a comparison study, rates of demethylation were determined for lamina and midrib of *N. glauca*, *N. otophora*, and a *N. tabacum* x *N. otophora* hybrid. Lamina had up to 3 x greater demethylation activity on a fresh weight basis than midrib tissue. Young leaves had greater activity than older leaves. This is significant since almost all alkaloids are biosynthesised in the root and translocated via the xylem, primarily to the

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younger shoot tissues. Consequently, nicotine would not be demethylated readily until it reached the leaves, site of accumulation. Lamina of *N. otophora* and the hybrid contained about 10 x more demethylase activity than lamina of *N. glauca. N. otophora* midrib contained about four times as much demethylase as *N. glauca* midrib.

Because of the high activity present, *N. otophora* was used to further characterize *in vivo* nicotine demethylation. *In vivo* nornicotine production could be detected after a 15 min incubation (Figure 1) and the rate of formation was nearly constant (~ 1 nmole h^{-1} mg^{-1}) up to 180 min. Incubation for 24 h resulted in conversion of 90% of the added 2'-^{14}C-nicotine to nornicotine. Nicotine demethylation in lamina was a linear function of incubation temperature in the range of 4 to 35°C.

A Lineweaver-Burke plot indicates that the demethylation process is saturable with an apparent K_m of 1 mM and V_{max} of 1.3 nmoles h^{-1} mg^{-1}. Data obtained in this laboratory with microsomal preparations of *N. otophora* lamina yielded a much lower apparent K_m of 119 μM. The discrepancy between the *in vivo* and *in vitro* values could be due to the *in vivo* presence of higher levels of nornicotine, a competitive inhibitor of nicotine demethylation Chelvarajan et al., 1991). Changing the pH of the incubation medium over the range of 5 to 8.5 showed a slight enhancement of enzyme activity in the pH 6 to 7.5 range.

Nicotine demethylation in *N. glauca* roots was inhibited 30% by incubation with 10 μM tetcyclasis and 56% by 20 μM tetcyclasis. Since tetcyclasis is a known inhibitor of cytochrome P-450 linked enzymes, these results suggest that a P-450 may be involved in the demethylation (Rademacher et al., 1987).

In an attempt to induce nicotine demethylation in *N. glauca*, hydroponically-grown plants were treated with phenobarbital, a N-demethylation inducer (P-450 enhancement) in mammalian liver and Jerusalem artichoke (Nakayama, 1988; Fonne-Pfister et al., 1988). In one experiment, root feeding of phenobarbital to *N. glauca* was associated with a marked increase in nicotine demethylation 2 days after treatment but a subsequent induction attempt was not successful. These observations may be similar to variations observed with phenobarbital induced nicotine metabolism in animals (Nakayama, 1988).

Nicotine demethylation in *N. otophora* root cortex was not inhibited by CO levels as high as 80% (20% O$_2$) but was inhibited by 100% CO. This is similar to observations made with rabbit liver microsomes (Murphy, 1973). Light did not reverse the inhibition by 100% CO. It is possible that the failure of light to reverse CO inhibition may be due to a relatively slow penetration of externally supplied nicotine (substrate limiting) rather than CO inhibition of oxygen binding to the cytochrome.

Nicotine demethylation increases with leaf maturation in *N. sylvestris* but not in *N. tabacum*. Lamina of *N. tabacum* and *N. sylvestris* exhibited similar low levels of demethylase activity before treatment with ethylene. Demethylase activity in lamina of *N. tabacum* did not change in control or ethylene-treated leaves during the 7-day experiment. However, in *N. sylvestris* an ethylene induction effect was evident on the 2nd day after the start of treatment (Figure 2). Demethylase levels in the ethylene-treated lamina increased 2.2 to 2.9 times as quickly as in control lamina.

Tobacco consumption has been largely attributed to presence of nicotine (Leffingwell, 1976) and the presence of nornicotine is undesirable (Smeeton, 1987). An understanding of the biochemical and molecular basis of expression of nicotine demethylase and the regulation (inhibition) of expression in tobacco would be desirable. These results suggest that in some *Nicotiana* species the enzyme for nicotine demethylation is present in non-nicotine accumulating tissues, is inducible, is inhibited by tetcyclasis and is most likely a P-450 dependent enzyme.

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LEGENDS

Figure 1. Nornicotine formation from ^{14}C-nicotine by small disks of *N. otophora* lamina.

Figure 2. Ethylene induction of nicotine demethylation in detached leaves of *N. sylvestris* and *N. tabacum*. Error bars indicate standard deviation of means of 2 or 3 experiments.

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Figure 1

% Nornicotine (y-axis, 0 to 100)

Hours of incubation (x-axis, 0 to 24)

Figure 2
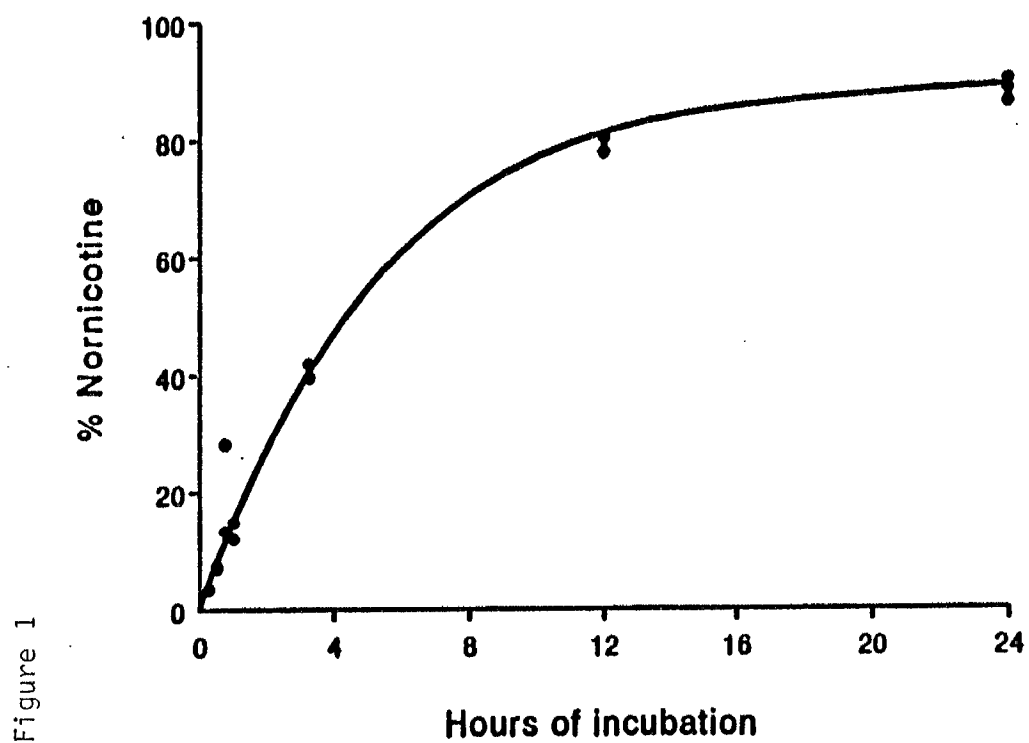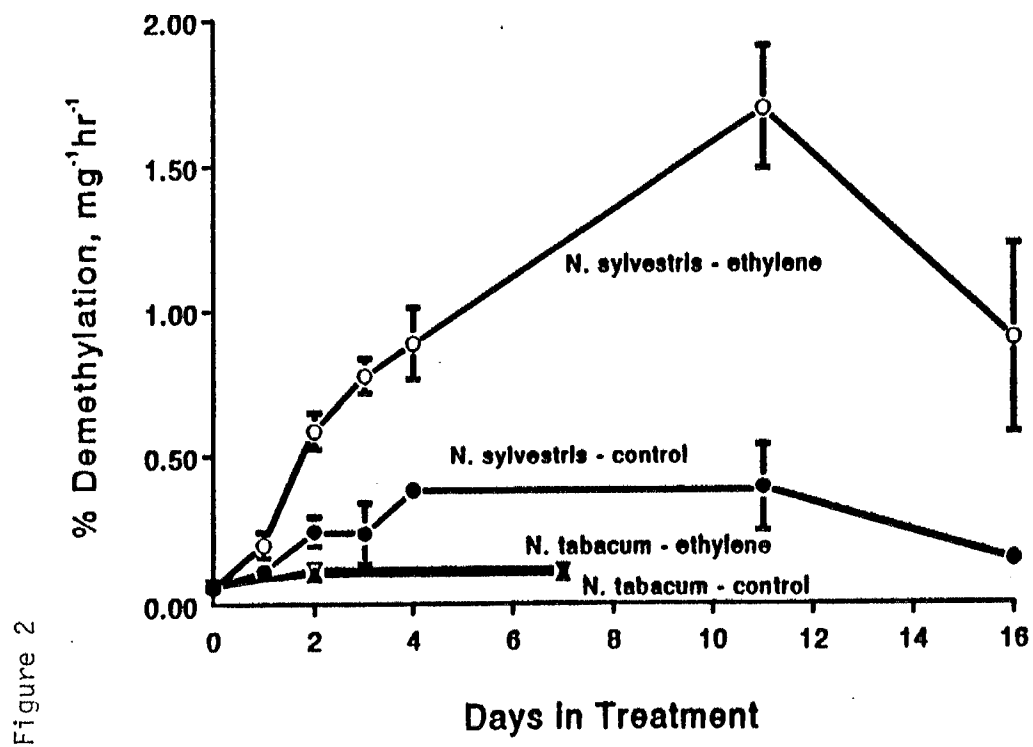
Study of Nicotine Demethylation in *Nicotiana otophora*

Nornicotine is a major alkaloid in *Nicotiana* and is the principal alkaloid in 30-40% of the species (Bush and Crowe, 1983). Virtually all nornicotine is formed from N-demethylation of nicotine (Dawson, 1951; Bush, 1981; Leete, 1984), and depending on the species, much of the nornicotine accumulation in *Nicotiana* occurs as the leaf matures (e.g., *N. otophora*) (Dawson, 1945) or during curing (e.g., *N. tabacum*) (Wada, 1956).

The demethylation of nicotine has been described to be controlled by one of two dominant genes (Griffith et al., 1955). This demethylation is not specific for the naturally occurring stereoisomer of nicotine, and partial racemization takes place during the process (Kisaki and Tamaki, 1961). The demethylation of nicotine as the primary source of nornicotine was first reported over 45 years ago by Dawson (1945). Nevertheless, the enzyme catalyzing this process has not been characterized or isolated. Schröter (1966) did detect *in vitro* nicotine demethylation in extracts from *N. alata*, but he did not present any *in vitro* properties of the enzyme catalyzing this process.

The objective of this study was to characterize the *in vitro* properties of nicotine demethylation from *N. otophora*.

Materials and Methods

Plant Material. *N. otophora* readily converts nicotine to nornicotine (Saitoh et al., 1985) and was used as the enzyme source. Lamina or pith was obtained from 6-month-old plants grown in a greenhouse with supplemental light from high-pressure sodium lamps.

Preparation of Microsomes. Microsomes were isolated at 4°C by a modification of methods used for the extraction of microsomes from higher plants (Reichhart et al., 1980; Dohn and Krieger, 1984; Mougin et al., 1990). Pith or lamina tissue was disrupted separately in a Waring blender in the presence of 8 volumes of N-(2-hydroxyethyl)piperazine-N'-[2-ethanesulfonic acid] (HEPES) buffer (50 mM, pH

7.5) supplemented with 250 mM sucrose, polyvinylpolypyrrolidone (pvpp) (0.2g/g of tissue), and 15 mM mercaptoethanol. The crude extract was filtered through two layers of cheesecloth to remove undisrupted tissue, and the filtrate was centrifuged for 20 min at 10000g to remove cellular debris. The supernatant was subjected to further centrifugation at 100000g for 60 min to pellet the microsomal fraction. The microsomal pellet was resuspended with the aid of a tissue homogenizer in 3 volumes of HEPES buffer (50 mM) supplemented with glycerol (30% v/v) and was stored in aliquots of 200 μL at -80°C.

Nicotine Demethylation Assay. Nicotine [pyrrolidine-2-^{14}C] was obtained from NEN Research Products and had a specific activity of 42.4 mCi/mmol. Routine enzyme assay consisted of approximately 80 μg of resuspended microsomal protein, 20 μmol of NADPH, and 10 μmol of nicotine and was made up to 40 μL with 50 mM HEPES buffer of pH 7.5. NADPH was omitted for control incubations. The reaction was carried out at 30°C for 30 min and was stopped with addition of 40 μL of 95% methanol, which also contained 50 mM nornicotine and nicotine to increase the recovery of [^{14}C]nornicotine and [^{14}C]nicotine. The amount of demethylation was measured using the thin-layer chromatography procedure of Fannin and Bush (1992).

Cytochrome P-450 Reductase Assay. The cytochrome P-450 reductase activity was determined by observing the reduction of cytochrome c with NADPH at 550 nm (Dohn and Krieger, 1984).

Oxygen Exclusion. Two treatments were carried out to determine if nicotine demethylation had a requirement for oxygen. The reaction components were dispensed into six 2.5-mL vials which were then hermetically sealed. Four vials were flushed with nitrogen (60 mL/vial), and two of these were then flushed with air (60 mL/vial). The two control vials were not flushed with either gas.

Solubilization. A number of detergents were screened for their ability to solubilize active nicotine demethylase from the microsomal fraction. About 100 μL of microsomal extract was incubated with 33.3 μL of various concentrations of detergent. The control had no detergent. The detergent-protein mixture was incubated at room temperature for 20 min and then centrifuged for 60 min at 100000g. An aliquot of the

supernatant was carefully removed and assayed for nicotine demethylase activity. The supernatant was then discarded, and the pellet was resuspended in buffer with the aid of an automatic dispensing pipet and then assayed for enzyme activity.

Results and Discussion

Powdering excised leaf tissue in liquid nitrogen and then extracting the soluble proteins with buffer (Frear et al., 1969) resulted in yields of active microsomal preparations that varied greatly between preparations. Omitting the liquid nitrogen step and disrupting tissue in 8 volumes of extraction buffer increased the yield and reduced the variability. Browning of the crude extract could not be prevented by the addition of 0.2 g/g PVPP or by a similar concentration of Amberlite XAD-4. As 15 mM mercaptoethanol did prevent browning, the extraction was performed in the presence of mercaptoethanol.

A small amount of demethylation was detected in the supernatant from the 100000g centrifugation. The supernatant activity was not diminished by incubating the microsomal preparation in the absence of NADPH, at 4 or 55°C, or by boiling the extract for 2 min prior to assaying. Hence, it was concluded that the demethylation associated with the supernatant was not enzymatic and was not characterized further. Although Schröter (1966) did not present any data on the *in vitro* characteristics of the demethylation of nicotine, the activity he measured may have been the nonenzymatic activity we observed in the 100000g supernatant. Schröter's activity too was located in the supernatant.

Microsomes stored for up to 5 months at-80°C were still able to catalyze nicotine demethylation. Unlike the enzymes catalyzing the N-demethylation of substituted 3-(phenyl)-1-methylureas in cotton (Frear et al., 1969), nicotine demethylase was not inactivated after one freeze-thaw cycle, but instead increased by up to 80%.

The level of microsomal N-demethylase in *N. otophora* [10-50 pmol min^{-1} (mg of protein)$^{-1}$], as measured by its capacity to demethylate nicotine, was considerably higher than N-demethylases from 12 species of higher plants. The levels of these N-

demethylases [<8 pmol min^{-1} (mg of protein)$^{-1}$] were measured by their ability to demethylate substituted 3-(phenyl)-1-methylureas (Frear et al., 1969). However, the rate of N-demethylation of aminopyrine by *Helianthus tuberosus* microsomes [186 pmol min^{-1} (mg of protein)$^{-1}$] (Fonne-Pfister et al., 1988) was considerably higher than the observed rate of nicotine demethylation in *N. otophora*.

A comparison of the level of NADPH-dependent cytochrome P-450 reductase in our *N. otophora* microsome with the level in higher plant microsomes prepared by other laboratories might indicate whether our procedure for microsomal preparation needed to be modified so as to optimize the *in vitro* yield of nicotine demethylation activity. The reductase activity in *N. otophora* was approximately 370 nmol min^{-1} (mg of protein)$^{-1}$, which was over 3-fold higher than the rate in microsomes from aging *H. tuberosus* tubers (Fonne-Pfister et al., 1988), suggesting that our protocol was adequate for microsome preparation.

Nicotine demethylation activity was dependent on microsomal protein concentration in the assay (Figure 1). Increased protein, up to 2 mg/mL, resulted in increased nicotine demethylation, but higher concentrations resulted in decreased activity; consequently, the concentration of microsomal protein used in assays was always less than 2 mg/mL. Inhibition by high microsomal protein concentrations was also observed in the demethylation of p-chloro-N-methylaniline (PCMA) in *Persea americana* (Dohn and Krieger, 1984).

The effect of temperature on nicotine demethylation was observed by incubating aliquots of the assay at various temperatures between 15 and 40°C. The temperature profile showed a maximum at 30°C and a 60% loss in activity by 40°C. All subsequent assays were conducted at the optimum of 30°C, which was also the optimum temperature reported by Dohn and Krieger (1984) for the demethylation of PCMA.

The pH profile, determined with 50 mM 2-(N-morpholino)ethanesulfonic acid (MES) and HEPES buffers, indicated that very little demethylation occurred below pH 6.0 (Figure 2). Optimum pH was between 7.0 and 7.5, which was very close to the pH 7.5 value reported by Fonne-Pfister et al. (1988) for aminopyrine N-demethylase in

phosphate buffer from *H. tuberosus*. Routine assays for nicotine demethylation were performed in 50 mM HEPES of pH 7.5.

Initially a phosphate assay buffer (100 mM, pH 7.0) was utilized since it was effective for the N-demethylation of substituted 3-(phenyl)-1-methylureas (Frear et al., 1969). When nicotine demethylation was measured in 50 mM HEPES buffer during the pH study, the rate of demethylation at pH 7.0 was over 3-fold higher than with phosphate. Magnesium too was found to be inhibitory, with a concentration of 10 mM resulting in a 50 % reduction in activity. Hence, demethylation was measured in HEPES without added magnesium.

A number of treatments were carried out on the 100000g pellet in an attempt to solubilize active nicotine demethylase. The detergents tested were CHAPS, glycodeoxycholate, cholate, octyl β-glucopyranoside, octyl β-thioglucopyranoside, sodium lauryl sulfate, and Triton X-100. High salt (300 mM KCl) and pH (pH 10) were also tried. Only Triton X-100 appeared to be successful in solubilizing nicotine demethylase as activity was observed in the supernatant after the detergent-protein mixture was centrifuged at 100000g for 60 min (Figure 3).

Nicotine demethylation required the presence of a reduced pyridine nucleotide. The rate of demethylation was approximately 15-fold faster with NADPH than with NADH. At low concentrations of NADPH, the rate of demethylation was greatly enhanced by NADH (Figure 4). Neither 10 mM NAD nor 10 mM NADP supported any demethylation, and in the presence of NADPH they were significant inhibitors (54% and 15% of control, respectively). In the absence of NADH, competing reactions might utilize NADPH, resulting in limiting concentrations of NADPH for nicotine demethylation. However, NADH might be preferentially oxidized in the presence of NADPH and thus spare NADPH for the demethylation reaction.

Since the rate of demethylation was linear for only the first 30 min, assays were stopped after 30 min. Beyond 30 min the reaction rate decreased rapidly and was nearly zero after 4 h. In an attempt to find the cause for this decrease in the rate of demethylation with time, various combinations of the assay components were incubated at 30°C for 1 h before further combinations of assay components were

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added and the reactions stopped after another 1-h incubation. Control was the incubation of all three components (NADPH, nicotine, and microsome) for 2 h with extra buffer added after 1 h (Figure 5a). Incubating only microsomes for 1 h before adding NADPH and nicotine (Figure 5b) resulted in nornicotine production of approximately half that of the control (Figure 5a). Incubation of microsomes in the presence of NADPH for 1 h did not result in any demethylation in the second hour after addition of nicotine (Figure 5c) . If nicotine was added with extra NADPH after 1 h of incubation of microsome and NADPH, demethylation of nicotine was observed (Figure 5d), indicating that when the microsomes were incubated with only NADPH, the NADPH was probably consumed by competing reactions during the first hour and some nicotine demethylase denatured. Incubating microsomes and nicotine for 1 h before adding NADPH (Figure 5e) produced one-third of the nornicotine of the control (Figure 5a) but significantly more than when the microsomes were incubated with NADPH (Figure 5c). Incubating NADPH and nicotine together prior to adding microsomes (Figure 5f) yielded nornicotine levels comparable to the control (Figure 5a). Extra microsomes added after 1 h did not result in further demethylation because NADPH and probably nicotine were limiting (data not shown). Also, extra nicotine or nicotine and microsomes did not enhance the production of nornicotine over the control. Addition of extra NADPH or NADPH and microsomes resulted in 18% and 26%, respectively, more enzyme activity over the 2-h period. Addition of all three components after the first hour resulted in 74% additional production of nornicotine in the second hour compared to the first hour. These results suggest that at 30°C the stability of nicotine demethylation was increased by the presence of both nicotine and NADPH.

Increased concentration of nornicotine (up to 50 mM) in the assay decreased the rate of nicotine demethylation. The concentrations of nornicotine encountered in routine assays ranged from 12.5 to 100 μM, and at these concentrations nornicotine did not inhibit nicotine demethylation. To determine if the nornicotine inhibition was a general alkaloid effect, an experiment was carried out with various concentrations of nicotine and nornicotine but with the total concentration at 2 mM. Nornicotine was a

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more effective inhibitor of demethylation than identical concentrations of nicotine. Hence, this inhibition was more likely a type of product inhibition.

Nicotine concentration was varied from 0 to 2 mM with the NADPH concentration kept constant (10 mM), to estimate K_m and V_{max}. From a Hanes plot of the resulting rates of demethylation, the apparent K_m for nicotine and V_{max} were estimated to be 51 μM and 24 pmol min^{-1} (mg of protein)$^{-1}$, respectively. The kinetic study was repeated with a different batch of microsomes, keeping the concentration of nicotine constant (0.33 mM) while varying the NADPH concentration from 0 to 4.8 mM. From a Hanes plot of the resulting rates, the apparent K_m for NADPH and V_{max} were estimated to be 5.75 mM and 105 pmol min^{-1} (mg of protein)$^{-1}$, respectively. The comparatively high apparent K_m for nicotine is mitigated by the high concentration of nicotine encountered in tobacco. At least one other N-demethylase, that demethylating PCMA in *P. americana*, has a K_m for PCMA (200 μM) (O'Keefe and Leto, 1989) significantly higher than the nicotine demethylase K_m for nicotine.

The involvement of cytochromes P-450 in the oxidative demethylation of several substrates has been demonstrated in higher plants (Reichhart et al., 1980; Higashi et al., 1985), and a number of tests were carried out to determine if this is true for nicotine demethylation. The heme containing cytochrome P-450 can also use hydroperoxides to catalyze demethylation (Estabrooke et al., 1984; Hollenberg et al., 1985). Neither hydrogen peroxide nor cumene hydroperoxide could substitute for NADPH and oxygen in nicotine demethylation, and in the presence of NADPH and oxygen, both cumene hydroperoxide (>0.15 mM) and hydrogen peroxide (>4 mM) actually inhibited demethylation.

Flushing vials with nitrogen completely inhibited the demethylation of nicotine, but if the nitrogen were immediately removed by flushing with air, activities comparable to the control values were obtained. These results indicate that the inhibition by nitrogen was purely due to oxygen depletion.

Tetcyclasis, an inhibitor of cytochrome P-450 type enzymes (Canivenc et al., 1989), at 60 μM inhibited nicotine demethylation by 50%. However, in wheat

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microsome a concentration of only 10 μM resulted in an inhibition of over 80% of cytochrome P-450 dependent aryl hydroxylase activity (McFadden et al., 1989).

Carbon monoxide also inhibits P-450 enzymes, and this inhibition can be reversed by light of 450 nm (Donaldson and Luster,1991) . Sparging the reaction components with carbon monoxide for 60 s (Omura and Sato, 1967) caused a reduction in nicotine demethylation activity of only 20% in lamina microsomes and no reduction in pith microsome (Figure 6). White light (2000 μEinstein m^{-2} s^{-1}) from a tungsten-halogen lamp did not reverse the slight inhibition observed in lamina microsomes, but further inhibited activity, possibly by interacting with the pigments present, resulting in the production of inhibitors to nicotine demethylation.

Various concentrations of a polyclonal antiserum raised in a rabbit against *H. tuberosus* NADPH-cytochrome P-450 reductase were added to the routine nicotine demethylase assay. The control was the incubation in the presence of a corresponding concentration of nonimmunized serum. Increased concentrations of antiserum increased inhibition of nicotine demethylation (Figure 7), suggesting a role for cytochrome P-450 reductase in the demethylation reaction. On the basis of their inhibition studies with the antiserum to cytochrome P-450 reductase, Benveniste et al. (1989) have concluded that cytochrome P-450 reductase is involved in reactions catalyzed by cytochromes P-450 in higher plants. Thus, if cytochrome P-450 reductase is involved in nicotine demethylation, this is a strong argument for the involvement of cytochrome P-450 as well.

Cytochrome P-450 dependent monooxygenases have previously been reported to be involved in higher plant alkaloid metabolism (Madyastha et al., 1976) and may also be involved in the demethylation of nicotine in *N. otophora*. Nicotine demethylation, like cytochrome P-450 mediated reactions, occurs in the microsomal fraction and is dependent upon molecular oxygen and reducing equipalents provided preferentially by NADPH and to a lesser extent by NADH. The stimulation of nicotine demethylation at subsaturating concentrations of NADPH by NADH is a characteristic of a number of cytochromes P-450 (West, 1980).

Although only partial inhibition by tetcyclasis and carbon monoxide suggests that cytochrome P-450 may not be involved in nicotine demethylation, these results do not rule out cytochrome P-450 involvement. Previous studies have indicated that the association between carbon monoxide and different cytochromes P-450 varies and can depend on the presence or absence of substrates (Tuckey and Kamin, 1983; Schröder and Diehl, 1987). It is also possible that tetcyclasis could have been bound to microsomal lipid, lowering the free concentration of tetcyclasis. At least one cytochrome P-450 dependent N-demethylase in wheat (Mougin et al., 1991) is known to be less sensitive to tetcyclasis than nicotine demethylase. The ability of peroxides to support demethylation in the absence of NADPH is not cited as a higher plant cytochrome P-450 characteristic by West (1980) but was considered in our study as certain cytochromes P-450 have been shown to be able to utilize peroxy compounds instead of NADPH (Estabrooke et al., 1984; Hollenberg et al., 1985). More cytochrome P-450 tests have to be carried out on the demethylation of nicotine before a definitive statement can be made on the role of cytochrome P-450, as not all of the primary criteria are satisfied by any one system (West, 1980).

Senescent leaves are characterized by a degradation of chlorophyll, carotenoids, starch, and chloroplast proteins (Long and Weybrew, 1981). Yet, the level of nornicotine in the leaves of numerous *Nicotiana* species rises during the last stages of curing (Wada, 1956). Other N-demethylases have also been reported to become more active during senescence (Fonne-Pfister et al., 1988). *in vivo* results from our laboratory have demonstrated greater levels of nicotine demethylation activity in leaves of *N. sylvestris* that were treated with ethylene to hasten senescence (Fannin and Bush, 1992). This suggests that the enzyme responsible for nornicotine synthesis is not degraded during early senescence but that the enzyme activity may be induced or activated during senescence.

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Figure to Legends

Figure 1. Relationship between microsomal protein concentration and demethylation. Protein concentration was measured using Coomassie[R] protein assay reagent.

Figure 2. pH profile of nicotine demethylase. pH was varied using MES (pH 5.0 to 6.5) and HEPES (pH 7.0 to 8.5) buffers. Data are shown with a spline curve fit.

Figure 3. Solubilization of nicotine demethylase from the microsomal pellett with Triton X-100.
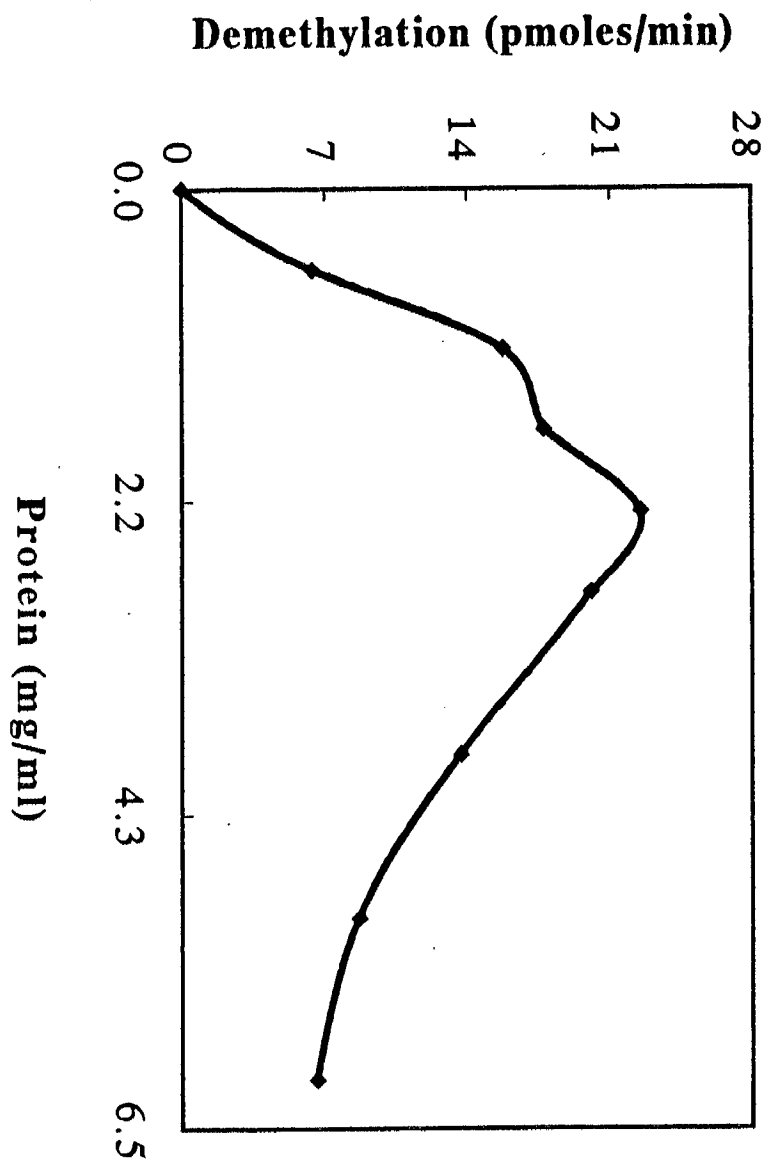
Figure 4. Effect of NADPH and HADH on nicotine demethylation.

Figure 5. Stability of nicotine demethylase at 30°C. Different combinations of microsomes, NADPH, and nicotine were incubated for 1 h. Further combinations of assay components were then added and the reactions allowed to proceed for an additional 1 h. The final volume for all incubations was 40 æl. The initial (1st hour) and final (2nd hour) incubations were as follows. (a) 1st: microsomes, NADPH, and nicotine; 2nd: extra buffer; (b) 1st: microsomes; 2nd: NADPH and nicotine; (c) 1st: microsomes and NADPH; 2nd: nicotine; (d) 1st: microsomes and NADPH; 2nd: NADPH and nicotine; (e) 1st: microsomes and nicotine; 2nd: NAPDH; (f) 1st: NADPH and nicotine; 2nd: microsomes.

Figure 6. Effect of carbon monoxide on demethylation. Nicotine demethylase activity was measured after subjecting the assay mixtures to: (a) light; (b) darkness; (c) carbon monoxide and light; (d) carbon monoxide and darkness. The microsomal preparation, NADPH solution, and assay buffer were sparged with 99.5% carbon monoxide for 45 s prior to incubation. The light source was a tungsten-halogen lamp, producing 2000 æE/m^2/s.
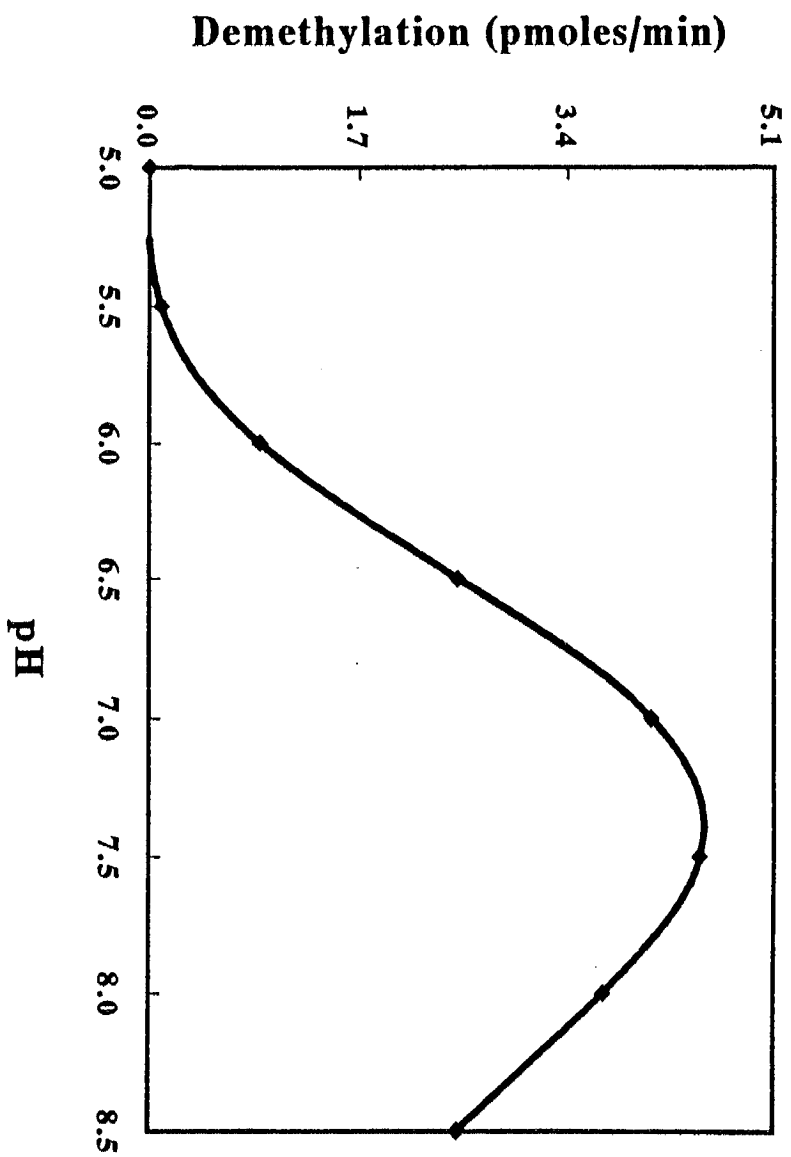
Figure 7. Effect of an antibody raised against *H. tuberosus* cytochrome P-450 reductase on the demethylation of nicotine.
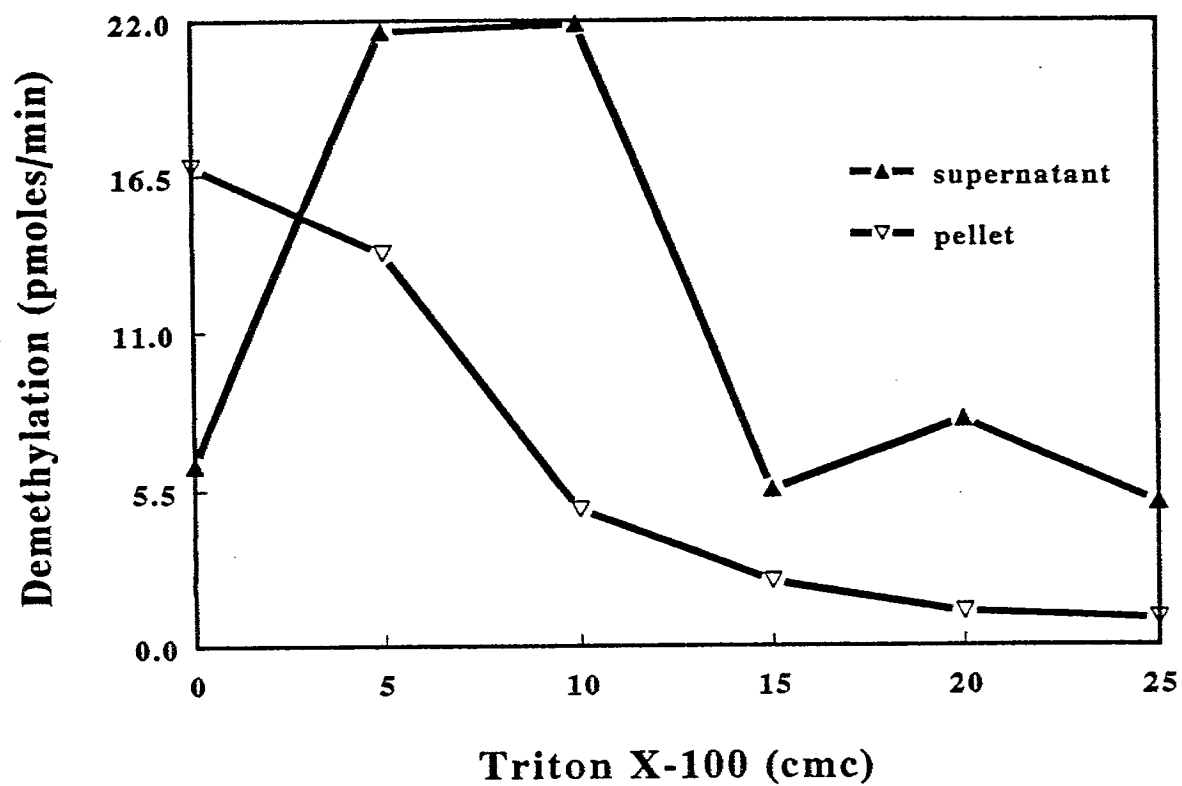
Figure 1
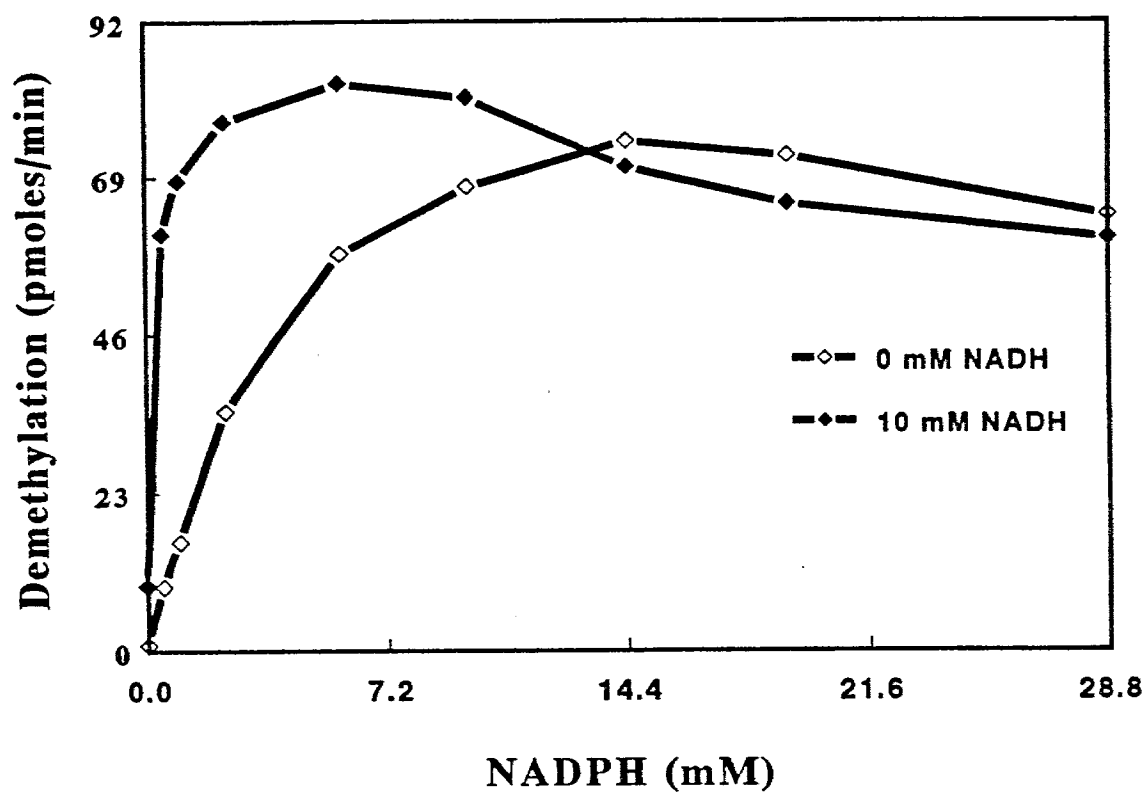
Demethylation (pmoles/min)

Protein (mg/ml)

Figure 2

Demethylation (pmoles/min)

pH

Figure 3

Figure 3 plots Demethylation (pmoles/min) on the y-axis (0.0, 5.5, 11.0, 16.5, 22.0) versus Triton X-100 (cmc) on the x-axis (0, 5, 10, 15, 20, 25). Two curves are shown: filled triangles (▲) for supernatant, and open inverted triangles (▽) for pellet.

Figure 4

Figure 4 graph: Y-axis "Demethylation (pmoles/min)" with values 0, 23, 46, 69, 92. X-axis "NADPH (mM)" with values 0.0, 7.2, 14.4, 21.6, 28.8. Legend: open diamond = 0 mM NADH; filled diamond = 10 mM NADH.

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Figure 5

Demethylation (% control)

Incubation

Figure 6

Demethylation (% control)

Figure 7

Antiserum (mg prot/mg microsomal prot)

Y-axis: Demethylation (% control), ranging 0 to 100
X-axis: 0.0, 1.5, 3.0, 4.5, 6.0

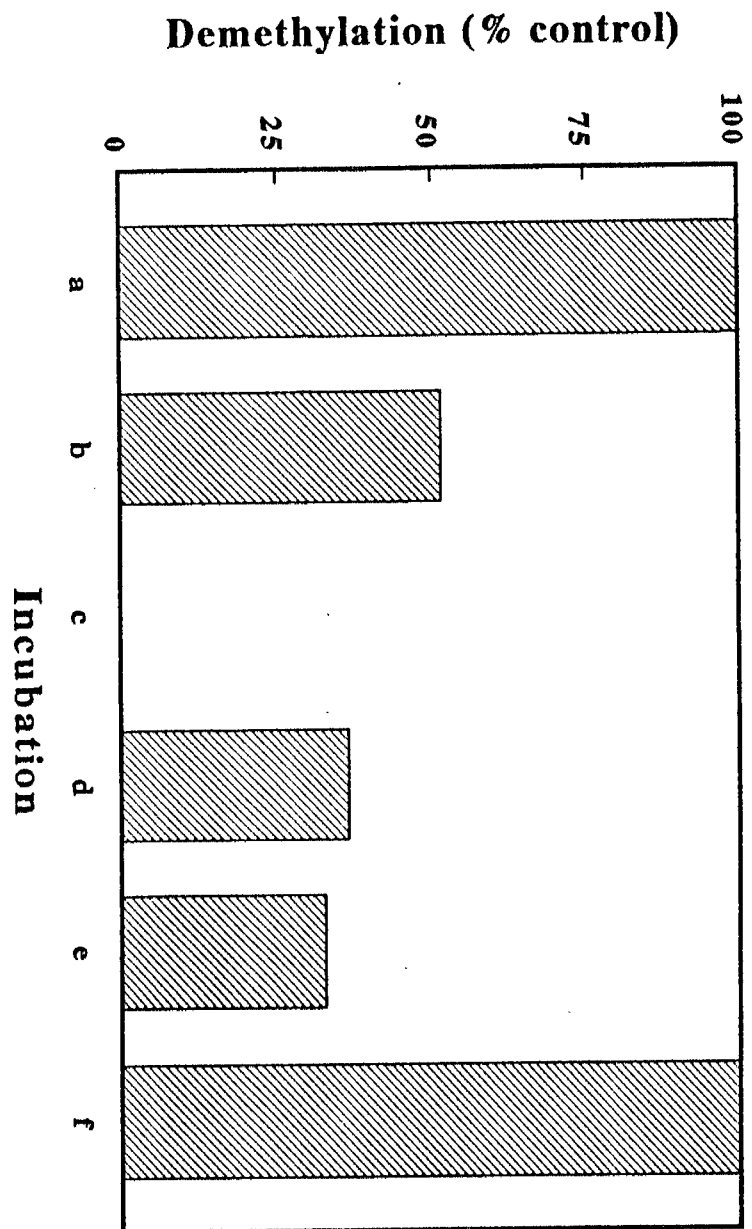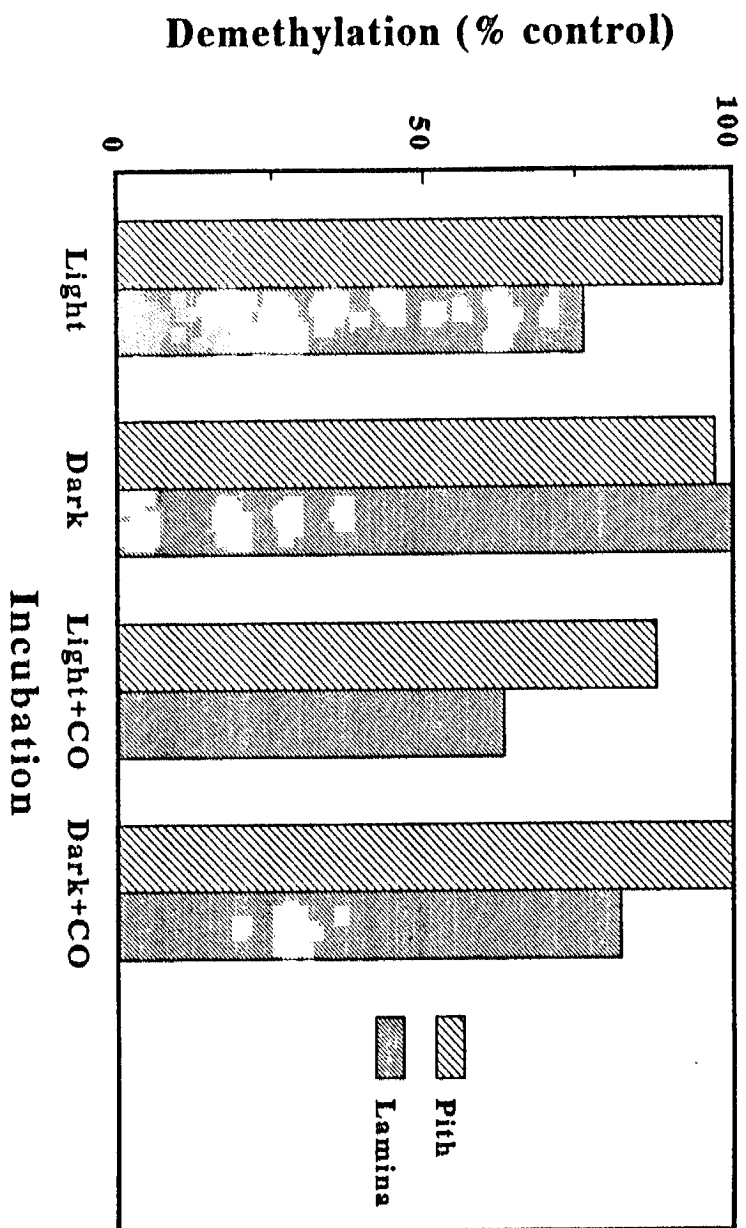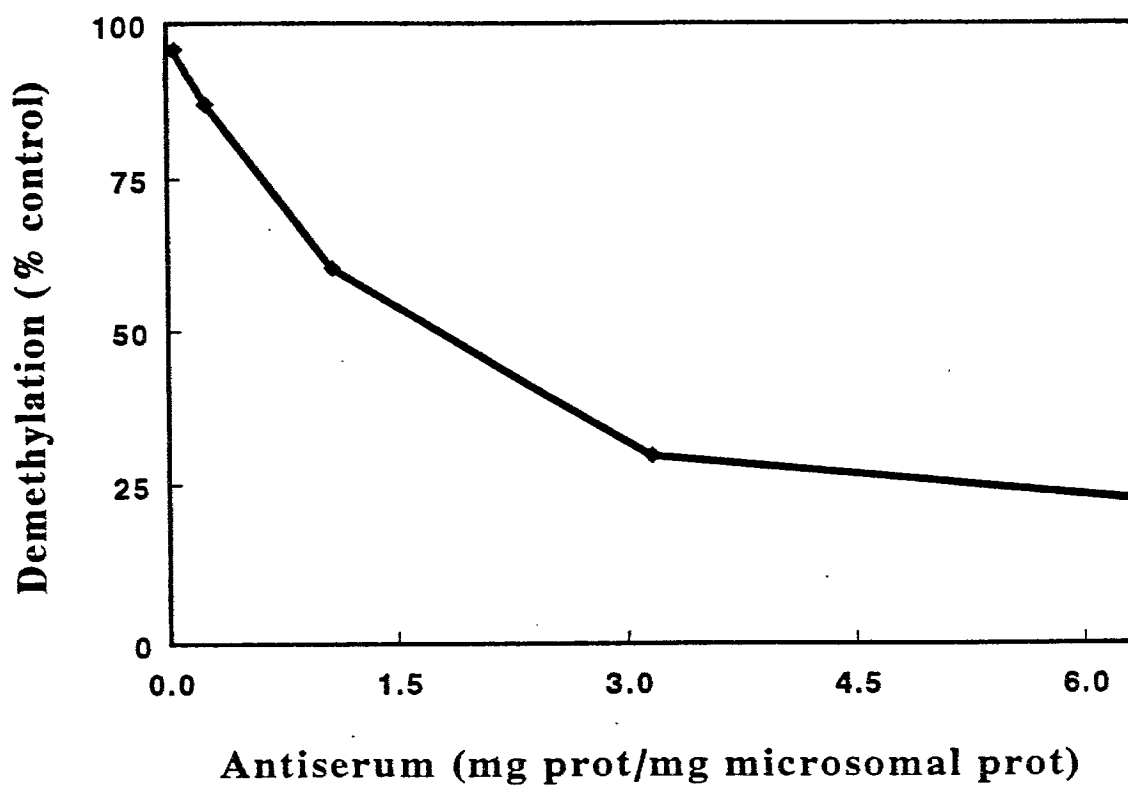## Influence of Rumen Fermentation on Response to Endophyte-Infected Tall Fescue Seed Measured by a Rat Bioassay

Tall fescue (*Festuca arundinacea* Schreb.) is an adaptable, cool season, perennial grass grown on approximately 35 million acres in the South Central United States (Lacefield and Evans, 1985). In spite of excellent agronomic characteristics, it is often less palatable and results in lower milk production, poorer weight gains and less overall productivity than similar cool season grasses (Stuedemann and Hoveland, 1988). This syndrome is referred to as "tall fescue toxicosis" and is caused by an endophytic fungus (*Acremonium coenophialum*) which often infects tall fescue. Lameness and gangrene of hind limbs and extremities can be observed in cold weather. During hot weather, body temperatures and respiration rates are increased above normal. Further symptoms are loss of body weight, emaciation, arched back, dull appearance, profuse salivation and a long rough hair coat. Alkaloid compounds have been associated with endophyte infection and tall fescue toxicosis (Hemken et al., 1984; Bush and Burrus, 1988). Three of the alkaloids present in tall fescue are the diazaphenanthrene alkaloids, the pyrrolizidine alkaloids and the ergotlike alkaloids. The diazaphenanthrene alkaloid, perloline, is found in both endophyte-infected and endophyte-free tall fescue. However, the pyrrolizidine and ergotlike alkaloids are only associated with endophyte-infected tall fescue.

Microbial fermentations in the ruminant forestomach have been shown to detoxify such diverse compounds as oxalate (Dawson and Allison, 1988), mimosine (Tagendjaja et al., 1983), 3-nitropropanoyl-D-glucopyranoses (Gustine et al., 1977), parathion (Cook, 1957), gossypol (Hungate, 1966) and digitalis (Hungate, 1966). In addition, some compounds are made more toxic in the rumen. Nitrates are converted to nitrites (Church, 1988), tryptophan is converted to 3- methyl-indole (Carlson and Breeze, 1984) and production of thiamin analogues in feedlot cattle results in polioencephalomalacia (Brent and Bartley, 1984).

Bio-assays using rats have been used to assess relative levels of toxicity. Peterson and Jago (1984) found that rats fed 20-40% Echium (containing pyrrolizidine

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alkaloids) had a 70% mortality rate. Neal and Schmidt (1985) found that rats fed up to 81% endophyte-infected fescue seed ate less feed, consumed less water, and grew at a slower rate than controls fed endophyte-free seed. Jackson et al. (1986; 1987; 1989) have also used rats to assess toxicity of tall fescue seed or various organic solvent extracts of tall fescue seed. Since rumen fermentation alters plant chemical constituents and can destroy or alter harmful compounds present in feedstuffs, then rumen fermentations may alter endophyte-infected tall fescue as well. This experiment used a rat model to assess possible alteration of the toxicity of tall fescue seed by incubation in rumen fluid for either 0 or 24 hours.

Materials and Methods

Twenty-eight male weanling Harlan Sprague-Dawley rats, weighing an average of 141 g, were randomly allotted to one of four treatments in a two*two factorial design. Treatments consisted of endophyte-free Johnstone tall fescue seed (E-), or endophyte-infected Kentucky-31 tall fescue seed (E+) incubated with rumen fluid for 0 (NON) or 24 hours (INC). Rumen fluid was collected from a cannulated donor steer fed a diet containing 50% corn, 37.5% tall fescue hay, 7.5% soybean meal, 3.8% molasses and 1.2% vitamin and mineral supplements. Fescue seed (226.8 g) was placed in a six-liter Erlenmeyer flask and rumen fluid added to the three-liter level. Four batches per treatment were required to obtain sufficient incubated material. Non-incubated (NON) treatments were transferred to smaller plastic containers and frozen immediately after rumen fluid collection. INC treatments were stoppered with a gas release valve and placed in a 37°C water bath for 24 hours. After stoppers were removed, incubation slurry was transferred to small plastic containers and frozen immediately. Frozen samples were dried in a Iyophilizer (LAEtCONCO Model 12, Laboratory Construction Company, Kansas City, Missouri) and mixed 1:1 with a commercially available laboratory chow (Purina Mills Inc, St. Louis, Missouri). Final composition of the four experimental diets (Table 1) consisted of 50% lab chow, 39% fescue seed and 11% rumen contents (air-dry basis). Diets were analyzed for ADF and NDF (Goering and Van Soest, 1970) and Kjeldahl nitrogen (AOAC, 1980).

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Pyrrolizidine alkaloids were determined using a modified capillary gas-liquid chromatographic method (Kennedy and Bush, 1983) with phenylmorphiline as the internal standard. Ergot alkaloids were determined using a modification of the HPLC procedure of Yates and Powell (Yates and Powell, 1988) and quantified using peak height. The experiment consisted of a 14-day growth period with weights and feed consumption data determined on days 0, 5, 10 and 14. Neal and Schmidt (1985) also conducted a 14-day growth experiment with rats consuming up to 81% of their diets as infected seed, and Jackson et al. (1986; 1987; 1989) have conducted several experiments ranging from 14 to 21 days duration. Rats were housed in a small animal room at the University of Kentucky animal laboratories and given a 2-week adaptation period prior to feeding the experimental diets. The temperature was maintained at a thermoneutral 23°C. Rats were sacrificed by exsanguination on day 14 and blood samples collected. Serum was frozen and saved for serum alkaline phosphatase analysis. Alkaline phosphatase was analyzed by reflectance spectrophotometry using a KODAK EKTACHEM DT60 (EASTMAN KODAK, CO., Rochester, NY) analyzer. Data were analyzed as a completely randomized factorial design using the General Linear Models procedure of SAS (1986). Mean differences were evaluated using a protected F-test.

Table 1. Composition of Experimental Diets[a]

Component	E-NON	E-INC	Treatment[b] E+NON	E+INC
Dry matter	91.5%	89.9	89.4	89.5
Crude protein	19.8	22.3	20.0	23.0
ADF[c]	9.2	8.0	8.6	7.8
NDF[d]	16.3	15.7	16.8	15.5
Pyrrolizidine alkaloids, $\mu g/g$	0	0	1502	1650
Ergot alkaloids, $\mu g/g$	0	0	3.66	3.78

[a] Diets composed of 50% laboratory chow (93.3% dry matter, 24.1% crude protein), 39% fescue seed, and 11% rumen contents.
[b] See Materials and Methods section for explanation of treatments.
[c] ADF - Acid Detergent Fiber
[d] NDF - Neutral Detergent Fiber

Results and Discussion

Rats fed E- diets had greater feed intake (see Table 2), daily weight gain and more efficient conversion of feed (feed conversion defined as g of feed/g of gain) than did rats fed the E+ diets ($P < .01$). This is consistent with the results of Neal and Schmidt (1985) and Jackson et al (1986; 1987; 1989) who found depressions in both gain and growth when rats were fed endophyte-infected tall fescue seed. Incubating seed with rumen fluid for 0 (NON) or 24 (INC) hours had minor effects on intake, gain and feed conversion efficiency. Rats fed INC seed had greater feed intakes ($P=.10$) than those fed NON, however, daily gain and feed conversion efficiencies were similar among treatments. Rumen microorganisms influence the digestibility of fibrous feed components by breaking down cell wall components such as cellulose, hemicellulose and pectins into usable forms such as short-chain-length steam- volatile fatty acids. Acid detergent fiber (ADF) and neutral detergent fiber (NDF), indicators

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of cell wall availability, were lowered slightly when incubated in rumen fluid, however, the magnitude of change was small and did not result in improved performance in rats fed incubated material (see Table 1). Crude protein levels were above recommended levels for growing rats. The increased levels of crude protein in incubated treatments might have been due to timing of rumen fluid collections.

Table 2. Feed intake, daily gain and feed efficiency (Whole Plot Effects)

Treatment	Initial Weight	Final Weight	Average Daily Gain	Average Daily Intake	Feed Conversion
Endophyte					
E-	141.2g	225.8^a	6.04^a	22.7^a	3.8^a
E+	140.9	172.5^b	2.26^b	14.3^b	6.7^b
S.E.	1.14	2.34	1.14	0.26	0.30
Incubation					
NON	141.3	198.1	4.06	18.2^c	5.6
INC	140.8	200.1	4.24	18.8^d	4.9
S.E.	1.14	2.34	1.14	0.26	0.30

^{ab}($P < 0.01$) ^{cd}($P = 0.10$)

Rats fed the E+INC (endophyte-infected seed incubated in rumen fluid) treatments had higher ($P<.05$) daily gain (Table 3) feed conversion efficiency than did rats fed the E+NON treatment. Increases in daily and feed conversion efficiency due to incubation were absent in E-INC. E-NON rats tended to have higher daily gain and feed conversion efficiency than did E-INC rats (non significant). These results suggest

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that the improvement in growth rate due to incubation in E+INC fed rats may be due to alteration of toxic components in E+ due to incubation in rumen fluid.

Table 3. Feed intake, daily gain, and performance (Endophyte*Incubation)

Treatment	Initial Weight	Final Weight	Average Daily Gain	Average Daily Intake	Feed Conversion
E+NON	141.3g	168.7[a]	1.96[a]	14.0[a]	7.5[a]
E+INC	140.6	176.3[a]	2.56[b]	14.6[a]	5.9[b]
E-NON	141.0	227.6[b]	6.16[c]	22.4[b]	3.6[c]
E-INC	141.3	223.9[b]	5.92[c]	23.0[b]	3.9[c]
S.E.	1.61	3.31	0.20	5.05	0.42

[abc]($p < 0.05$)

Period means for daily feed intake, gain and feed conversion are presented in Figures 1 and 2. Means for endophyte and incubation level are presented in Figure 1 (main effects) and interaction effects between endophyte and incubation level are presented in Figure 2. Rats consuming E- diets consumed more feed in all three periods and gained faster during the first two periods than did rats consuming E+ diets ($p < .01$). Rats consuming E+ diets actually lost body weight during the first five day period. Rats consuming E+INC diets consumed more feed and either lost less weight (day 0-5) or gained more weight than E+NON rats during all three periods. There were no significant interactions in any of these periods, although gain and feed conversion were significant ($p < .05$) over the 14-day period as previously noted (see Table 3).

It is interesting that rats appeared to be adapting to endophyte consumption over the 14-day period. From negative gains in the first five days, E+ rats adapted to

gain similar amounts as E- rats at the end of the 14-day period. However, feed intake of E+ rats never reached that of E- rats. Growth rate of E- rats declined at the end of the trial as weight plateaued. Feed intake for E- rats indicates there were no consumption problems when adding rumen fluid or endophyte-free tall fescue seed to the chow based diet. Intake on E- diets was similar to that of chow fed controls in our lab and consistent with NRC (1978) values.

Serum alkaline phosphatase values are reported in Table 4. Previous research has indicated that alkaline phosphatase concentrations are depressed in cattle fed endophyte- infected fescue (Smith, 1986) and is often used as a diagnostic measurement of fescue toxicosis. Rats fed infected fescue seed (E+ diets) had significantly higher levels of alkaline phosphatase (P<.01). In addition, fescue seed incubation (INC) also resulted in a significant rise in alkaline phosphatase levels (P<.05). There were no endophyte*incubation interactions observed (P>.10). These results indicate a potential species difference between rats and cattle. Concentrations in these rats were higher than previously reported values (Kramer, 1989). The levels of both pyrrolizidine and ergot alkaloids present before and after 24-hour incubation (1502 and 1650,ug/g of pyrrolizidine alkaloids and 3.66 and 3.78, μg/g of ergot alkaloids) for E+NON and E+INC, respectively, are similar and provide no indication of alkaloid compounds being destroyed during the 24-hour incubation. Bush et al. (1970; 1972; 1976) found that in vitro cellulose digestibility was depressed by added perloline (diazaphenanthrene alkaloid found in tall fescue). However, perloline effects were probably limited in this experiment because perloline is present in tall fescue seed in only very small quantities (Gentry, 1968). Any effects due to incubation do not appear to be mediated through destruction of these particular alkaloids and may be

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due to destruction of some as yet unidentified alkaloids present in endophyte-infected tall fescue.

Table 4. Serum alkaline phosphatase levels

	Levels μg/l
Endophyte-infection	
E-	565.0[a]
E+	785.4[b]
S.E.	29.8
Incubation	
NON	628.3[c]
INC	722.0[d]
S.E.	29.8
Endophyte*Incubation	
E+NON	705.6[c]
E+INC	865.1[c]
E-NON	551.1[d]
E-INC	578.9[d]
S.E.	42.2

[ab]$(P < 0.01)$ [cd]$(P < 0.05)$

Rasmussen et al. (1989) adapted sheep to a diet containing 50% *Lathyrus sylvestris* (Flatpea) for up to four months. Lathyrus contains the potentially toxic amino acid derivative L-2,4-diaminobutyric acid (DABA). After adaptation they exchanged rumen contents from sheep fed flatpea or alfalfa. The formerly flatpea

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adapted sheep became sensitive, and the formerly flatpea naive sheep became tolerant after transfer. This showed that adaptation had occurred and destruction of the toxin was taking place. Adaptation of ruminants to toxic materials has been shown by other researchers as well. Dick et al. (1963), Lanigan and Smith (1970), and Lanigan (1970) have all reported on the ability of rumen microorganisms to destroy the hepatotoxic pyrrolizidine alkaloids heliotrine and lasiocarpine. Lanigan (1976) isolated the microbe, Peptococcus heliotrinreducans, which is important in catalyzing these detoxifying reactions. Open monoester and diester pyrrolizidines are metabolized in ovine rumen fluid resulting in the conversion of the open ester pyrrolizidines to 1-methyl derivatives (Cheeke, 1988). The closed or cyclic ester pyrrolizidines are not detoxified in the rumen. The resistance of sheep to pyrrolizidine alkaloid toxicity results from a slow liver pyrrole production from both the open and closed esters as well as rumen detoxification of the open esters.

The major compounds implicated in tall fescue toxicosis are the pyrrolizidine alkaloids N-acetyl and N-formyl loline, and the ergot alkaloids (Stuedemann and Hoveland, 1988; Bush and Burrus, 1988). In this research, rats fed endophyte-infected fescue seed incubated with rumen fluid gained faster than those fed non-incubated fescue seed; however, there were no concomitant decreases in either pyrrolizidine or ergot alkaloid concentrations. Ball et al. (1957) reported that rats fed autoclaved tall fescue gained more weight than rats fed tall fescue seed which had not been autoclaved. Our results indicate that the toxic components in tall fescue can be destroyed with a corresponding increase in growth rate.

The results presented here confirm those of previous researchers (Neal and Schmidt, 1985; Jackson et al., 1986; 1987; 1989). Rats fed endophyte-infected tall fescue seed ate less, gained less, and converted feed less efficiently than controls fed

endophyte-free seed. Incubation of endophyte-infected tall fescue seed in rumen fluid increased daily gain and feed conversion efficiency in our rat model.

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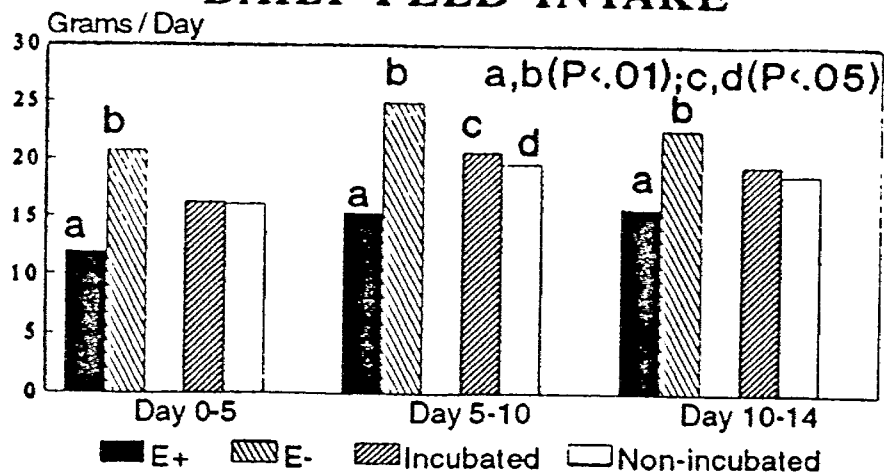
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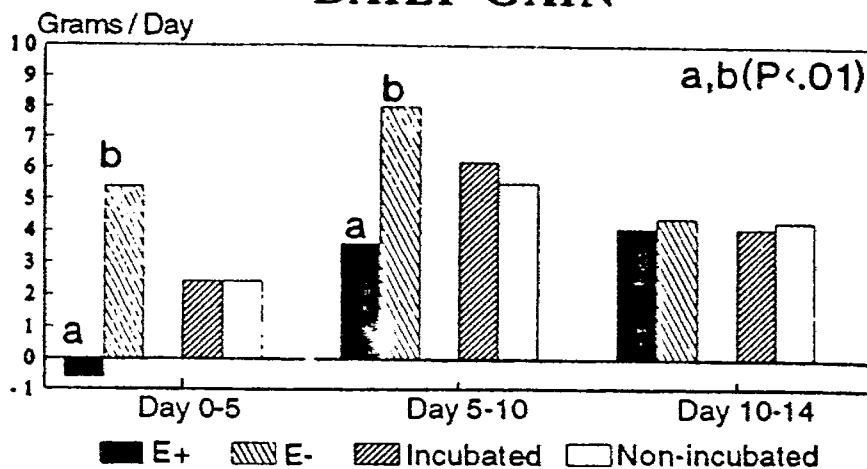2055007413

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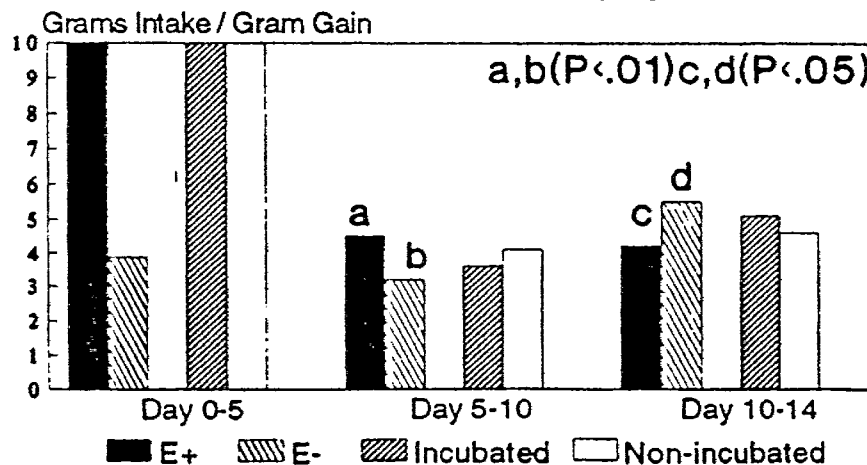
DAILY FEED INTAKE

85

Grams / Day

a,b(P<.01);c,d(P<.05)

b

b

c   d

a   a   a   b

Day 0-5   Day 5-10   Day 10-14

■ E+   ▨ E-   ▨ Incubated   □ Non-incubated

DAILY GAIN

Grams / Day

a,b(P<.01)

b

b

a

a

Day 0-5   Day 5-10   Day 10-14

■ E+   ▨ E-   ▨ Incubated   □ Non-incubated

FEED CONVERSION

Grams Intake / Gram Gain

a,b(P<.01)c,d(P<.05)

a   d

b   c

Day 0-5   Day 5-10   Day 10-14

■ E+   ▨ E-   ▨ Incubated   □ Non-incubated

Day 0-5 feed conversion not figured

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DAILY FEED INTAKE

DAILY GAIN
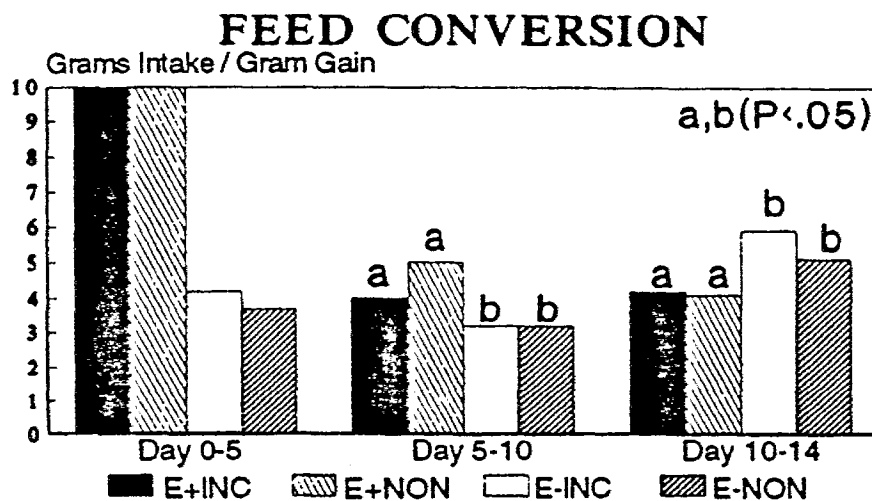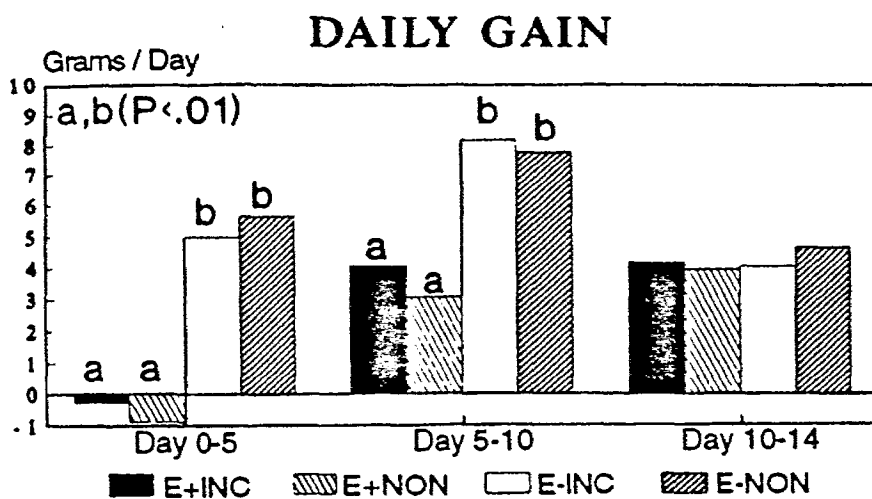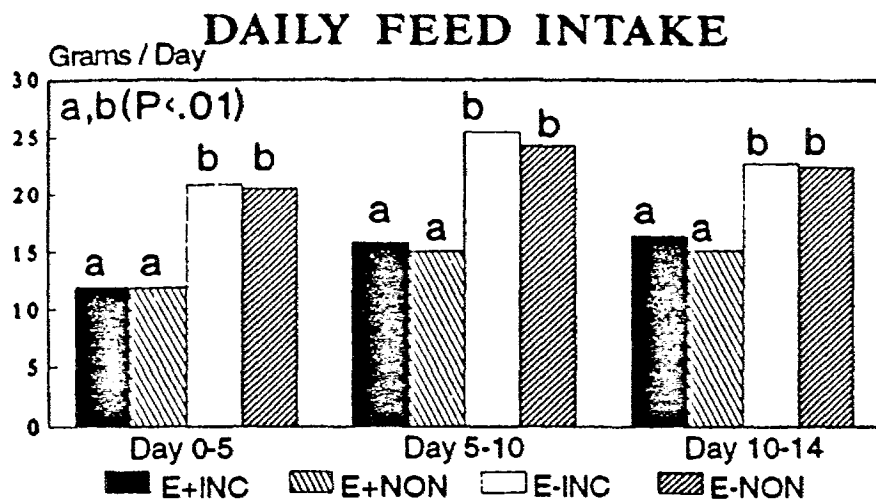
FEED CONVERSION

E+ day 0-5 feed conversion not figured

FIGURE 2

In Vitro and In Vivo Ruminal and Physiological
Responses to Endophyte-Infected Tall Fescue

Tall fescue toxicosis affects cattle grazing tall fescue during hot summer months. It is characterized by poor BW gains, intolerance to heat, salivation, increased body temperatures and respiration rates, rough hair coats, poor milk production, and reduced conception rates, causing severe economic losses for cattle producers throughout the southeastern United States. Tall fescue toxicosis is associated with the presence of the endophytic fungus, *Acremonium coenophialum*, which infects tall fescue and produces alkaloid compounds that may mediate toxicosis. Three classes of compounds present in tall fescue are the diazaphenanthrene alkaloids, the ergot alkaloids, and the pyrrolizidine alkaloids. The diazaphenanthrene alkaloids are associated with both endophyte-free (EF) and endophyte-infected (EI) tall fescue; the ergot and pyrrolizidine alkaloids are associated with EI tall fescue alone. All of those compounds are suspected toxins. Perloline has negative effects on in vitro and in vivo ruminal metabolism (Boling et al., 1975; Bush et al., 1970). Symptoms of fescue toxicosis are similar to those of ergotism (Bacon et al., 1986), and, because tall fescue contains detectable ergot alkaloids, ergot alkaloids may be the principal alkaloids involved in tall fescue toxicosis. The pyrrolizidine alkaloids, N-formyl loline (FL) and Nacetyl loline (AL), inhibited the mitogenic growth of blood lymphocytes (Hayek et al., 1991), and Johnson et al. (1985) demonstrated their toxicity to plant parasites. Although structurally related, FL and AL are chemically and physiologically different from pyrrolizidine alkaloids occurring elsewhere in nature (Cheeke, 1988).

Previous research has shown that compounds with potent toxicological or pharmacological activity are degraded in the ruminant forestomach (Dawson and Allison, 1988). Mimosine (Tagendjaja et al., 1983), glucopyranose (Gustine et al., 1977), and oxalate (Dawson et al., 1980) are examples of compounds degraded in ruminal fluid cultures. In addition, researchers (Cheeke, 1988; Lanigan, 1976) have studied the effects of ruminal metabolism on degradation of some naturally occurring pyrrolizidine alkaloids. However, no estimates of ruminal metabolism have been made

for the alkaloids present in EI tall fescue. If these alkaloids are destroyed in the rumen, then less should be available for absorption. This research was completed to determine the extent of in vitro alkaloid degradation when different combinations of the pyrrolizidine alkaloids, FL, and AL, are incubated in ruminal fluid. Also, reported is research using sheep equipped with abomasal sampling cannulas and an inert ruminal flow marker to determine alkaloid recovery. The influence of dietary concentrations of alkaloids on diet digestibility also was studied.

Materials and Methods

Experiment 1

Treatments (four replications per treatment) consisting of seven different combinations (Table 1) of FL and AL were added to incubation vials containing .5 g of an EF fescue diet (8% cracked corn, 8% soybean meal, 78% 'Johnstone' tall fescue hay, 5% molasses, and 1% vitamin and mineral supplements), 25 ml of ruminal fluid (from a steer consuming the same diet), and 25 ml of McDougall's buffer solution (McDougall, 1948) and incubated in a 37°C shaking water bath for 0, 24, or 48 h. All techniques were performed anaerobically. Combination treatments contained FL and AL in approximately the same ratio (1:2, wt/wt) found in EI tall fescue. Purified alkaloids were prepared courtesy of the USDA-ARS (Peoria, IL) by the procedure of Petroski et al. (1989). All samples were frozen, dried to a constant weight in a lyophilizer (Virtues, Gardiner, NY), and analyzed for FL and AL contents using a modified capillary GLC procedure (Kennedy and Bush, 1983) with phenylmorphiline as the internal standard. Loline formation also was measured by a capillary GLC procedure (Yates et al., 1990) and quantitated by peak height. All results were corrected to 100%, time 0 alkaloid recovery. Presence of microbial fermentation was verified by measurement of gas production. Results were analyzed as a splitplot design; FL and AL alkaloid recovery was the whole plot, and time of sampling was the subplot using the general linear models procedure of SAS (1985). Replication within treatment sum of squares served as the whole-plot error term and residual sum of squares was the subplot error term. Time effects were analyzed using orthogonal

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polynomials; repeated measures analysis was used for verification. When time was significant, treatments were separated within time period using Bonferroni's t statistic.

Table 1. N-Formyl and N-acetyl loline alkaloid treatments.

Treatments[1,2]	Alkaloid Concentrations		
	Total Alkaloid	N-Acetyl	N-Formyl
	(mg per vial)[3]		
Control	0.0	0.00	0.00
1AL	1.0	1.00	0.00
1FL	1.0	0.00	1.00
1AL plus FL	1.0	0.33	0.67
5AL	5.0	5.00	0.00
5FL	5.0	0.00	5.00
5AL plus FL	5.0	1.65	3.35

[1] Control = 0 added alkaloid; 1AL = added N-acetyl loline; 1FL = added N-formyl loline; 1AL plus FL = N-acetyl plus N-formyl added in combination; 5AL = added N-acetyl loline; 5FL added N-formyl loline; 5AL plus FL = N-acetyl plus N-formyl loline added in combination.
[2] AL plus FL treatments contain N-acetyle (AL) and N-formyl (FL) loline in a 1:2 ratio similar to that found in tall fescue.
[3] Amount in milligrams added to each incubation vial.

Experiment 2

Twelve Hampshire cross wethers (58 kg of BW), fitted with abomasal collection cannulas, were allotted to a completely random arrangement of treatments and placed on their respective diets (Tables 2 and 3). Wethers were fitted with abomasal cannulas (Precision Machine Co., Lincoln, NE) using surgical procedures described by Komarek (Komarek, 1981). Postsurgical antibiotic treatment consisted of 400,000 units of procaine penicillin G and .5 g of dihydrostreptomycin intramuscularly for 4 d following surgery.

Table 2. Composition of diets.

Component	EF[1]	EI945	EI2346
	(%, as-fed basis)		
Ground fescue hay			
'Johnstone' (EF)	51	51	51
Fescue seed			
'Johnstone' (EF)	40	20	0
'Kentucky-31' (EI)	0	20	40
Ground corn	3	3	3
Molasses	4	4	4
Cr_2O_3	0.5	0.5	0.5
Trace-mineralized salt[2]	0.5	0.5	0.5
Limestone	1	1	1
Vitamin premix[3]	0.04	0.04	0.04

[1] EF = Endophyte-free diet; EI945 = endophyte-infected diet containing 945 µg of N-formyl plus N-acetyl loline alkaloids/g of diet; EI2346 = endophyte-infected diet containing 2346 µg of N-formyl plus N-acetyl loline alkaloids/g of diet.

[2] Formulation of trace-mineralized salt contains not more than 98.5% NaCl; contains not less than 0.95% ZnO, 0.35% $FeCO_3$, 0.34% Mn_3O_4, 0.2% FeO, 0.033% mineral oil, 0.007% Cu_4O, or 0.005% $Ca(C_{18}H_{35}O_2)_2$; and contains 55 ppm of $Na_2SeO3 \cdot 5H_2O$ and traces of $Ca(IO_3)_2 \cdot 6H_2$), $CoCO_3$, and natural and artificial flavors.

[3] Vitamin premix containing 8,818,342 IU of vitamin A, 1,763,668 IU of vitamin D, and 1102 IU of vitamin/kg.

Table 3. Chemical analysis of diets.

Constituent	EF[1]	EI945	EI2346
	(%, as-fed basis)		
DM	91.59	91.66	91.58
CP	12.63	12.69	12.93
NDF	50.07	50.98	47.09
ADF	21.74	20.37	20.45
Total,[2] μg/g	0	788	1955
N-formyl	0	576	1471
N-acetyl	0	212	484
Ergot alkaloids, μg/g	0	1.4	1.6

[1] EF = Endophyte-free diet; EI945 = endophyte-infected diet containing 945 μg of N-formyl plus N-acetyl loline alkaloids/g of diet; EI2346 = endophyte-infected diet containing 2346 μg of N-formyl plus N-acetyl loline alkaloids/g of diet.
[2] N-Formyl plus N-acetyl loline alkaloids.

'Johnstone' (EF) and 'Kentucky-31' (EI) tall fescue were used as sources of EF and EI seed. Chromic oxide was used as an inert marker and added to the diets using a ground corn premix. Actual alkaloid (FL plus AL) intakes were 0 mg (EF), 945 mg (EI945), and 2346 mg (EI2346)/d per sheep on the respective treatments. Sheep were adapted to diets for 10 d. During the adaptation period, all sheep were placed in metabolism crates and fed at 0800 and 2000 h. Intake was equalized at 1200 g/d per sheep. At the end of the adaptation period, 6-d samples were collected as described by Hays et al. (1964). Approximately 70 ml of abomasal fluid and a fecal grab sample were collected at each time. All samples were lyophilized in a Virtues lyophilizer and composited by weight (except when inadequate sample precluded an equal composite). Orts were weighed back after each feeding, and water intake was measured daily. Rectal temperatures and respiration rates were taken on d 2, 4, and 6; results presented are the average of three measurements. Blood samples were taken on d 5, and serum was separated and frozen until later analysis for alkaline phosphatase.

Feed, digesta, and fecal samples were analyzed for DM, ADF, NDF, CP, FL, AL, and the ergot alkaloids, ergovaline and ergovalinine. Both ADF and NDF were analyzed using the method of Goering and Van Soest (1970). Kjeldahl N was determined using AOAC (1980) procedures. Contents of FL and AL were determined as described previously. Ergot alkaloids were determined using a modification of the HPLC procedure of Yates and Powell (1988); quantitation was by peak height, and results were expressed as total ergots (ergovaline plus ergovalinine). Chromic oxide was determined using the spectrophotometric method of Hill and Anderson (1958). Estimates of nutrient digestibility and alkaloid disappearance were made using chromic oxide:nutrient ratios. Serum samples were analyzed for alkaline phosphatase using a Multistat III Micro Centrifugal Analyzer™ (Eastman Kodak, Rochester, NY).

All data, except alkaloid recovery, were analyzed as a completely randomized design; orthogonal contrasts were used to separate treatment means. The following contrasts were made for diets: EF versus (EI945 and EI2346), and EI945 versus EI2346. Alkaloid recovery was analyzed as a split-plot design; treatment was the whole plot, and site of sampling was the subplot. Replication within treatment sum of squares was the error term for separating treatment means, and residual sum of squares was the error term for separating site of sampling means. Site of sampling means were separated using orthogonal contrasts. Contrasts made were dietary alkaloid recovery versus recovery in digesta (abomasal contents plus feces) and abomasal contents versus feces recovery. All statistical procedures were carried out using the general linear models procedure of SAS (1985).

Results and Discussion

Results of Experiment 1 are presented in Tables 4 and 5. Gas production (Table 4) increased ($P < .01$) over the 48-h incubation period, confirming microbial activity. Time zero alkaloid recovery averaged 88% for all treatments and was higher for 1-mg (97%) than for 5-mg (77%) treatments. The reasons for those differences are unclear, but differences may have resulted from analytical error or from spontaneous deformylation or deacylation when ruminal fluid was added to incubation vials. A

quadratic (P < .05) effect was present of time on recovery of total alkaloids, FL, and AL over the 48-h incubation (Table 4). Repeated measures analysis also indicated a time response (P < .01) and a time by treatment response (P < .01). Because of this time by treatment effect, data were analyzed within time periods to determine treatment differences (Table 5). No treatment differences occurred in alkaloid recovery after 24 h (P > .20). However, after 48 h, recoveries of FL and FL plus AL combinations were significantly less than AL recovery (P < .001). Not only was FL more readily degraded than AL, but its presence also decreased recovery of AL in combination treatments.

Table 4. Influence of incubation time on in vitro alkaloid recovery and gas production.

Alkaloid[1]	Recovery			SE
	0 h	24 h	48 h	
	(%)			
Total[a]	100.0	81.3	37.6	3.8
AL[a]	100.0	91.7	58.3	4.8
FL[a]	100.0	72.1	3.1	4.7
Loline[b]	0.0	4.0	8.3	0.9
Gas produced, ml[c]	0.0	66.4	97.3	0.8

[a] Quadratic effect of time on percentage of alkaloid recovery (P < 0.05).
[b] Linear effect of time on percentage of loline alkaloid recovery (P < 0.01).
[c] Quadratic effect of time on gas production (P < 0.05).
[1] Total = N-Formyl plus N-acetyl loline; FL = N-formyl loline; AL = N-acetyl loline.

Table 5. Influence of incubation time and alkaloid treatment on alkaloid recovery.

Treatment[1,2]	Recovery				
	AL and FL recovery			Loline alkaloid appearance	
	0 h	24 h	48 h	24 h	48 h
	(%)				
Control	0.0	0.0	0.0	0.0 [c]	0.0 [c]
1AL	100.0	95.1	106.0 [a]	0.0 [d]	0.0 [d]
1FL	100.0	68.4	0.0 [b]	11.4 [cd]	8.7 [d]
1AL plus FL	100.0	86.6	2.7 [b]	6.1 [c]	10.0 [d]
5AL	100.0	86.0	96.4 [a]	0.0 [c]	0.0 [c]
5FL	100.0	77.4	8.3 [b]	4.7 [cd]	18.0 [d]
5AL plus FL	100.0	72.2	10.2 [b]	1.8 [cd]	13.3 [d]
SE		13.2	8.3	2.8	4.0

[a,b] AL different from FL and AL + FL treatments ($P < 0.001$).

[c,d] AL different from FL but not AL + FL treatments ($P < 0.05$).

[1] Control = 0 added alkaloid; 1AL = added N-acetyl loline; 1FL = added N-formyl loline; 1AL plus FL = N-acetyl plus N-formyl loline added in combination; 5AL = added N-acetyl loline; 5FL added N-formyl loline; 5AL plus FL = N-acetyl plus N-formyl loline added in combination.

[2] AL plus FL treatments contain N-acetyl (AL) and N-formyl (FL) loline in a 1:2 ratio similar to that found in tall fescue.

Quadratic time effects and repeated measures analysis indicate that degradation increased over the 48-h incubation. Microbial adaptation to substrates often is characterized by a lag phase of growth (Hungate, 1966). This lag phase is a period when little growth occurs prior to microbial adaptation. Rate of alkaloid degradation may increase after this adaptation is complete. Readily fermentable substrates also may have been depleted, and the microbial population began using alkaloids as an energy source during the second 24 h.

Metabolism of FL and AL and conversion to the alkaloid loline increased as incubation increased (Table 5), but only in FL or FL plus AL combinations. Conversion to loline accounted for only a portion of total disappearance. In contrast, no conversion was observed on either AL treatment. No conversion to other pyrrolizidine alkaloid derivatives, such as norloline or N-methyl loline, was observed.

Results of Experiment 2 are presented in Tables 6, 7, and 8. Table 6 compares the influence of three levels of endophyte infection on nutrient digestibility, rectal temperature, respiration rate, and water intake. Digestibility of all components measured was less for EI dias than for the EF diet. Total tract DM digestibility ($P < .10$), ruminal and total tract ADF and CP digestibilities ($P < .05$), and ruminal NDF digestibility ($P < .06$) were highest on the EF diet. The EI945 diet appeared to maximize these effects. Total tract DM and ADF digestibilities were higher in sheep fed the EI2346 diet than in those receiving the EI945 diet. Depressed digestion is in agreement with observations of Hannah et al. (1990) and Fiorito et al. (1991), who also found depression in digestibility when sheep and lambs consumed EI tall fescue. No differences existed in rectal temperature between EF and EI diets ($P > .10$). Mean rectal temperature (39.63°C) of sheep fed the EI2346 diet was higher than for those of sheep fed the EI945 diet (39.47°C). Although significant, this small difference is of doubtful biological significance. No differences were significant between treatments in respiration rate, water intake, or serum alkaline phosphatase concentrations. Smith (1986) found depressed concentrations of alkaline phosphatase in cattle consuming EI tall fescue. However, Fiorito et al. (1991) found no differences in alkaline phosphatase concentration of lambs consuming low and high endophyte diets. The alkaline phosphatase concentrations in the present study are much lower than those previously reported.

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Table 6. Influence of endophyte on diet digestibility, water intake, rectal temperature, respiration rate, and serum alkaline phosphatase.

Response	Diet[1] EF	Diet[1] EI945	Diet[1] EI2346	SE
Daily DMI, g/d	1199	1200	1199	--
Digestibility, %				
DM[a,b]	73.1	69.5	72.2	0.9
ADF				
Ruminal[c]	45.9	31.2	34.8	3.8
Total tract[d,e]	60.4	52.9	57.5	1.6
NDF				
Ruminal[f]	59.4	51.3	49.6	3.1
Total tract	71.2	67.5	69.5	1.3
CP[g]	75.8	73.0	74.5	0.6
Alkaline phosphatase, μg/L	5.3	21.0	26.0	12.4
Rectal temperature, °C[h]	39.46	39.47	39.63	0.04
Respirations/min	43.7	47.3	46.0	10.5
Water intake, L/d	3.2	2.8	2.9	0.3

[a] EF versus EI differ $(P < 0.10)$.
[b] EI945 versus EI2346 differ $(P < 0.10)$.
[c] EF versus EI differ $(P < 0.05)$.
[d] EF versus EI differ $(P < 0.05)$.
[e] EI945 versus EI2346 differ $(P < 0.10)$.
[f] EF versus EI differ $(P < 0.06)$.
[g] EF versus EI differ $(P < 0.06)$.
[h] EI945 versus EI2346 differ $(P < 0.05)$.
[1] EF = Endophyte-free diet; EI945 = endophyte-infected diet containing 945 ug of N-formyl loline plus N-acetyl loline alkaloid/g of diet; EI2346 = endophyte-infected diet containing 2346 ug of N-formyl plus N-acetyl loline alkaloid/g of diet.

Table 7. N-Formyl and N-acetyl loline alkaloid recovery.

Sampling site	Recovery			SE
	EI[1,2]	EI945	EI2346	
		(%)		
Diet[a]	NP	100.0	100.0	0.9
Abomasal digesta[b]	NP	5.5	4.3	0.9
Feces	NP	0.0	0.0	0.9
	Individual alkaloid recovery (abomasal)			
N-Formyl loline	NP	3.6	3.2	2.5
N-Acetyl loline[c]	NP	10.4	7.5	2.5

[a] Dietary alkaloid differs from abomasal digesta or feces ($P < 0.001$).
[b] Abomasal digesta differs from feces ($P < 0.01$).
[c] Alkaloid effect ($P < 0.05$).
[1] EF = Endophyte-free diet; EI945 = endophyte-infected diet containing 945 μg of N-formyl plus N-acetyl loline alkaloid/g of diet; EI2346 = endophyte-infected diet containing 2346 μg of N-formyl loline alkaloid/g of diet, and NP = alkaloids not present in EF diets.

Table 8. Ergot alkaloid recovery.

Sampling site	Percentage of recovery			SE
	EF[1]	EI945	EI2346	
		(%)		
Diet[a]	NP	100.0	100.0	12.5
Abomasal digesta[b]	NP	61.8	47.1	12.5
Feces	NP	5.9	6.9	12.5

[a] Dietary alkaloid differs from abomasal digesta or feces ($P < 0.001$).
[b] Abomasal digesta differs from feces ($P < 0.01$).
[1] EF = Endophyte-free diet; EI945 = endophyte-infected diet containing 945 μg of N-formyl plus N-acetyl loline alkaloid/g of diet; EI2346 = endophyte-infected diet containing 2346 μg of N-formyl loline alkaloid/g of diet, and NP = alkaloids not present in EF diets.

Recovery of FL and AL alkaloids from abomasal and fecal samples of sheep fed EF or EI diets is summarized in Table 7. Recovery averaged 5% of dietary

administered alkaloids in abomasal contents (P < .001), and no alkaloids were detected in the feces. Recovery of AL averaged 10.4 and 7.5%, and recovery of FL averaged 3.6 and 3.2% for the EI945 and EI2346 diets, respectively. Chromatograms from pyrrolizidine alkaloid analyses indicated that loline increased in abomasal digesta samples, further confirming the conversion to loline in the in vitro experiment. The loline alkaloids recovered in abomasal digesta accounted for about 10% of the total FL and AL fed.

Table 8 shows ergot alkaloid recovery. Recoveries of ergot alkaloids were significantly (P < .001) reduced in the abomasal digesta, and concentrations in the feces were much lower (P < .01) than in abomasal contents.

Alkaloid disappearance was much greater in the sheep experiment. Over 90% of FL and AL alkaloids disappeared anterior to the abomasum and 100% prior to the feces, compared with only 30 to 40% disappearance after a 24-h in vitro incubation. Only after a 48-h incubation was alkaloid disappearance similar to that in the sheep experiment. As in the in vitro experiment, portions of FL and AL were converted to loline. Some of these differences may be explained by absorption from the rumen in the in vivo system, by a greater microbial adaptation time in vitro, the complexity of the ruminal environment, or a combination.

These experiments were intended to determine the relationships among EI tall fescue, alkaloid compounds produced by EI tall fescue, and biochemical processes occurring in the rumen. The in vitro alkaloid experiment provides evidence that alkaloids are degraded in ruminal fermentation. Disappearance of FL and AL increased over the 48-h incubation period. The greatest alkaloid disappearance occurred in the FL and FL plus AL combination treatments; the least disappearance occurred in treatments containing only AL. As disappearance of FL and AL increased, the appearance of loline increased. Appearance was greatest in those treatments containing FL; in treatments containing AL alone, loline was not present.

Chromatograms indicate that FL and AL alkaloids were converted to loline in vivo as well as in vitro; quantitation indicated conversion was extensive, particularly in the sheep experiment, which partially explains the difference between the two

experiments. These data indicate a conversion of FL and AL to loline during ruminal metabolism (Figure 1). The majority of this conversion apparently is from FL (the predominant pyrrolizidine alkaloid in tall fescue), but appreciable quantities of AL may be converted as well. Loline inhibited the mitogenic growth of blood Lymphocytes more than either FL or AL (Hayek et al., 1991). The conversion of relatively nontoxic compounds by ruminal microbes to more toxic compounds is known to occur, as in the case of tryptophan, which can be converted to 3-methyl-indole and is responsible for acute bovine pulmonary emphysema, or in the conversion of nitrate to nitrite during nitrate poisoning (Church, 1988).
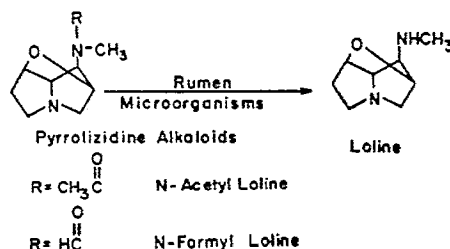

Figure 1. Pyrrolizidine alkaloid metabolism

In the sheep experiment, estimates of ergot alkaloid disappearance were also made. The fraction of ergot alkaloids (ergovaline and ergovalinine) that disappeared before abomasal collections was much less than for FL and AL alkaloids (between 40 and 50%). The relative stability of the ergot alkaloids toward ruminal bioconversion suggests that they may play a greater toxic role than the pyrrolizidine alkaloids in the etiology of tall fescue toxicosis because they remain available for postruminal absorption. However, which alkaloids have the greater effect is inconclusive from these data because of the possible ruminal absorption of FL and AL and because of the potential toxicity of loline, a ruminal metabolic product. The absence of FL, AL, or loline in fecal collections also supports possible postruminal absorption of these compounds.

In vitro studies by Bush et al. (1970) showed that perloline inhibited cellulose digestion and the growth of certain cellulose-degrading microbes, and an in vivo study by Boling et al. (1975) indicated that perloline decreased both CP and cellulose digestion. In the present experiment, ruminal and total tract ADF digestibility decreased, as did ruminal NDF digestibility, in sheep consuming EI tall fescue relative to controls. These measurements agree with similar results of Fiorito et al. (1991) and Hannah et al. (1990). However, Neal and Schmidt (1985) found increased digestibility of DM, crude fiber, and nitrogen-free extract when EI seed was fed to rats; and Goetsch et al. (1987) reported increased DM, NDF, and nitrogen digestibility as the EI material in the diet increased. Both Neal and Schmidt (1985) and Goetsch et al. (1987) allowed the ad libitum consumption of EI material to depress intake (relative to EF controls fed for ad libitum intake), which may be expected to increase digestibility. Intake was equalized in the present study and in studies by Fiorito et al. (1991) and Hannah et al. (1990), who also reported depressed digestibility of fibrous components.

Hannah et al. (1990) reported 17 and 15% reduction in cellulose and NDF digestibility, respectively, and Fiorito et al. (1991) reported 15 and 8% reduction in ADF and NDF digestibility, respectively, when sheep were fed EI diets. In the present study, total tract ADF and NDF digestibility (average of two EI treatments) were reduced relative to controls by 9 and 4%, respectively. Ruminal ADF and NDF digestibility (average of two EI treatments) were reduced 28 and 15%, respectively. It is unclear why digestibility in the EI2346 treatment increased relative to that in the EI945 treatment. Hannah et al. (1990) reported the greatest reduction in fiber digestibility in the treatments containing the greatest level of endophyte infection. Errors in sampling or in distribution of marker in the feed are possible explanations for the differences between these two treatments.

Ruminal metabolism is clearly influenced by EI tall fescue; however, which alkaloids mediate this effect is not known. Perloline has negative effects on fiber digestibility. However, perloline effects probably were limited in the present study because perloline is only present in very small quantities in tall fescue seed (Gentry, 1968). Because seed was used as the source of endophyte infection, it is unlikely that

digestibility depressions were mediated by perloline and more likely that they were related to one of the other toxic factors in EI tall fescue. Perloline also is present in only minute quantities in 'Johnstone' tall fescue hay. 'Johnstone' was the tall fescue cultivar used as a hay source in this study. Further research is required to determine which factors in tall fescue are responsible for the observed digestibility depression.

Conclusions

The results indicate that 1) both ergots and FL and AL alkaloids disappear from the rumen in vivo, 2) disappearance of FL is greater than that of AL, 3) FL is converted to loline, and 4) dietary digestibility is depressed by EI diets.

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Biosynthesis and Metabolism of Nicotine and Related Alkaloids

Alkaloids are extremely important in tobacco (*Nicotiana tabacum*) leaf because they are an important factor in smoke quality, and they provide a physiological stimulus which makes the use of tobacco products pleasurable. Nicotine and other alkaloids in *Nicotiana spp.* have been ascribed many functions - i.e., (1) detoxification products, (2) waste products, (3) nitrogen reserve, (4) regulatory substance for plant growth, and (5) protection for the plant against insects and other herbivores - and consequently they appear to be important to the evolution of the genus as it is known today. However, as important as *Nicotiana* alkaloids are in eliciting animal responses from the utilization of tobacco, growth and development of the plant proceed normally in the absence of accumulation of alkaloids.

Nicotine levels in tobacco are affected by genetics, environmental conditions and cultural practices. Influence of environmental conditions and cultural practices on nicotine accumulation in tobacco has been subject of many reviews (i.e. Bush and Saunders, 1977; Chaplin and Miner, 1980; Bush and Crowe, 1989). Genetic and physiologic aspects of tobacco alkaloid biosynthesis and accumulation was reviewed last by Leete (1980), Bush (1981), and Strunz and Findley (1985).

In the 60-plus species of *Nicotiana*, most alkaloids are 3-pyridyl derivatives with nicotine the principal alkaloid in 50 to 60% of the species. Based on amounts of alkaloid accumulation in leaves of *Nicotiana spp.* - nicotine, nornicotine, anatabine and anabasine - are the major alkaloids present in the genus. Nornicotine is the major alkaloid in 30 to 40% of the species. Anabasine is usually the major alkaloid in *N. acaulis, N. glauca, N. petuniodes* and *N. solanifolia.* Anatabine is usually not the principal alkaloid in any species but will be a relatively higher percentage of the total in *N. otophora, N. tomentosa* and *N. tomentosiformis.* Species that accumulate primarily nicotine tend to have higher total alkaloid concentration in their leaves. There are many minor alkaloids found in tobacco leaves which are derivatives of the major alkaloids and some of these are shown in Figs. __.1 and __.2. Most of the minor alkaloids are present in less than 50 μg g^{-1} dry weight and many are present in

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nanogram amounts. Many of these alkaloids are apparently aberrant metabolism or minor catabolic products of the major alkaloids and even may be artifacts formed during isolation from tobacco.

Alkaloid Biosynthesis

The precursors for the pyridine, pyrrolidine, and piperidine rings of nicotine, nornicotine, anatabine, and anabasine have been determined and biosynthetic schemes developed that are consistent with the data; however, many of the intermediates in the biosynthetic sequence are not known nor have many of the enzymes required for the biosynthetic steps been fully characterized. Precursor and biosynthetic sequences are important to understanding alkaloid metabolism, but to manipulate leaf chemistry the metabolic regulation must be understood. Metabolic regulation of alkaloid accumulation should ideally be treated in both its qualitative and quantitative aspects, with consideration of alternate pathways and controls, as well as, variations in levels of enzyme activity and metabolic effectors. The knowledge of the biosynthetic and degradative pathways of alkaloids in tobacco that is needed for studies of this kind is still incomplete. Accordingly, the most accepted routes for alkaloid biosynthesis will be presented and then the role of significant enzymes and potential metabolic regulation will be discussed.

Nicotine. The pyridine ring of nicotine, nornicotine, anabasine and anatabine is formed from nicotinic acid. Quinolinic acid (pyridine-2,3-dicarboxylic acid) was an efficient immediate precursor of the nicotinic acid incorporated into the pyridine ring (Yang et al., 1965). They also showed that glycerol was incorporated without randomization into C-4, C-5, and C-6 of the pyridine ring. $3\text{-}^{14}C$-aspartate or $3\text{-}^{14}C$-malate fed to *N. rustica* resulted in the label being almost exclusively incorporated into C-2 and C-3 position of the pyridine ring (Jackanioz and Byerrum, 1966). All intermediates in this synthetic sequence have not been elucidated (Fig. ___.3).

Substantial evidence indicates that the major biosynthetic route for the N-methylpyrrolidine ring of nicotine proceeds through putrescine (1,4-diaminobutane), N-methylputrescine and 4-methylaminobutanal (N-methyl-4-aminobutyraldehyde) to the

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